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A place for science in the power game

As a highly respected scientific pressure group, Pugwash might reasonably have expected some publicity for the meeting it organised in London last week to discuss the fast breeder reactor. The advent of fast reactor technology is already becoming acknowledged as perhaps the major social issue of the decade in the developed world. In Britain, the Royal Commission on Environmental Pollution (RCEP) had placed the controversy firmly in the public spotlight with the publication of its report on nuclear power only six days before the Pugwash meeting. But Pugwash went unmentioned by the media. The meeting was ignored by the television corporations, passed unreported on the radio, and received not a single column inch in any of the national daily newspapers. It is worth considering why.

To some extent the unbroken media silence must be attributable to the very timeliness of the meeting, coming as it did so hard on the heels of the RCEP report. There is little press copy to be had from issues already extensively covered less than a week before. Admittedly, the meeting covered most of the contentious ground, and presented both sides of the argument. But it is worth considering whether the media's disregard might reflect more than just a fickle boredom with recurring issues. It could, for instance, say something about the role of Pugwash in the widening debate on the fast breeder. The part that it played in educating both scientists and politicians on nuclear matters during the 1960s stands as solid testimony to its influence at that time. But does it wield such influence still?

Pugwash itself apparently believes that it can still reach politicians by going through the back door. Last week's invitation list did not include the UK Energy Secretary, Anthony Wedgwood Benn, or his two political advisers. As for the House of Commons Select Committee on Science and Technology, members were invited, but it looked very much as though most, if not all, of the invitations were treated with disdain. Moreover, of the top personnel from the Department of Energy (DEN) only Walter Marshall could be seen, and his silence and early departure gave no clue as to whether he attended as Chief Scientist at the DEN, Deputy Chairman of the UK Atomic Energy Authority, or simply to assess any new opposition in the nuclear power game. Whatever his motives, the 350 or so scientists who confronted him offered little new beyond the contention that a target date of 1995 for a demonstration commercial fusion reactor in Europe could become realistic with more funding.

But in seeking to understand the lack of media interest not all of the blame can be apportioned to Pugwash and absent politicians. Many of the scientists present would do well to reflect what their true contribution to the fast breeder debate has been. For while it was encouraging to

note the number of well-known and respected members of the scientific community eager to discuss all the implication of fast reactor technology, and even prepared to talk seriously in the corridors of the untapped potential of alternative technologies, there remain far to many who see fast breeder reactors simply in terms of load factors, breeding ratios, doubling times, and the like. Consequently, it was left to the eloquent rhetoric of a former Minister of Fuel and Power, Philip Noel-Baker, to set the debate squarely in its political and social perspective, and by the time of his closing speech many of those who ought to have been listening had seen their fill of graphs and tables and dispersed to their buses and trains.

Such a cold approach by the science community makes a mockery of the frequent protests, voiced as loudly as ever during last week's meeting, against media misrepresentation of major science-based issues. For those members of the press whom Pugwash bothered to invite, the choice was simple and clear: they could either search for sensational angles, or settle for reporting a mundane and sometimes technically bewildering meeting exactly as it happened. Evidently, they responsibly chose to do the latter and ran up against their colleagues' more newsworthy competition. Which leaves science with a sad alternative: sensational media coverage (remember the Williams report on genetic engineering?), or no media coverage at all.

And that is the crunch. For though it has wide and inescapable political and social ramifications, the debate over the fast breeder reactor is firmly rooted in science, and it surely falls on the science community to ensure that the public and their politicians are kept intelligibly informed by an impartial, well-briefed press. Sir Brian Flowers, former Chairman of the RCEP and a board member of the UKAEA, recently stressed how the rules of technological advance have changed in the developed world: it is no longer sufficient for each new step forward to be technologically realisable unless it is also socially acceptable. It is in assessing that social acceptability that science must assume a crucial rôle.

Only three weeks ago it looked very much as though the powerful British nuclear lobby might, rightly or wrongly, force an early decision in favour of the UK's first demonstration commercial fast breeder, CFR-1. Now the RCEP, in giving the reactor only qualified approval in its new report, has won a delay of a few months at least for nuclear power critics. Mr Benn and the nuclear lobby have been pushed against the baseline, and the ball has fallen to those in the other court. For the rest of the match it is the scientific community that should act as umpire. And from that position it must ensure that whoever comes out on top, every single blade of grass is covered. □



Small isn't beautiful either

At the beginning of January 1976, one pound sterling would buy 2.02 US dollars. Last week you could get only \$1.66 for a pound; at one stage as little as \$1.63. This depreciation of nearly 20% in sterling is making for grim news almost everywhere in Britain; the laboratories of British universities will feel the pinch, for the Science Research Council (SRC) is almost certainly facing an acute financial crisis.

Within the past year the SRC, in common with many organisations dispensing government money, has had to accept cash limits on its activities, that is to say, it has been given a firm sum of money above which it may not commit itself. The SRC has also, of course, been told by the Advisory Board of Research Councils (ABRC) to trim its spending over the next five years by 10% in real terms. To achieve this, the so-called big sciences have been told that many major national projects cannot go ahead, and international facilities such as CERN, Institut Laue-Langevin and the European Space Agency have accordingly increased in importance. Subscriptions to these international organisations amount to about 30% of SRC's budget, and go up every

time the pound goes down.

So now, in order to fulfil its international obligations, the SRC is looking for £6 million more than it had budgeted for—with cash limits in operation to prevent it from simply going to the Treasury and asking for the difference. In fact the Treasury is prepared to sympathise to the tune of £2 million, so the SRC is left either to cut £4 million off domestic expenditure, to sweet-talk CERN into letting us pay less, or (the most likely) some combination of these two.

What can be done on the domestic scene? The SRC has relatively few options open, in that much of its money is committed for long-term projects already half-done. Vulnerable spots are therefore new grant proposals and new research studentships. The new crop of research students is barely large enough to satisfy demand; even some candidates with first-class degrees have not yet been placed, and eight hundred candidates with upper-second degrees are going to be disappointed. On the research grants side the scene is even grimmer. At the last round of grant-giving there were significant numbers of applicants who failed even

though their application had been rated in the top category. This had never happened before. One of the options that the SRC is bound to be considering is that the next round of proposals (there are three a year) is simply dropped altogether.

In the somewhat longer run, SRC may have to get down to the serious business of making some of its own staff redundant. It employs nearly 3,000 in six laboratories and headquarters. With generous redundancy terms to meet, the council is unlikely to benefit in the short term from such action, but it might be encouraged by the contents of a forthcoming Confederation of British Industry document 'The Road to Recovery' which, according to *The Economist*, will suggest that industry could create a million new jobs, into which a hundred well-qualified SRC employees could surely fit.

The irony of the SRC's present crisis is that if grant giving is seriously curtailed it will be the 'small sciences' which will suffer, in order to keep the big sciences going—hardly ABRC's intention when it looked to more use of international facilities by Britain's big scientists. □

More on Argentina

CONCERN about the plight of scientists in Argentina has grown steadily since we published a letter from Italian academics on the subject (July 22). It has become increasingly clear that a country whose scientific skills have been widely acknowledged and respected is in the greatest danger of abandoning its intellectual traditions and becoming a scientific backwater. The number of academics and workers in research institutes who have lost their jobs is growing all the time, and some fairly detailed documentation is emerging.

Inevitably, when left- and right-wing extremists are at loggerheads, some of those dismissed or detained will have had a history of political activity, and thus will be vulnerable to charges of using their position for political rather than academic purposes. But many others, relatively apolitical, seem to have been caught in the crossfire. When military politicians such as General Vilas can say that "until we can cleanse the teaching area, and professors are all of Christian thought and ideology we will not achieve the triumph we seek in our struggle against the revolutionary left", there is little hope that anything resembling academic freedom can survive.

Even the Atomic Energy Commission does not seem to have escaped the general purge. At least eight employees are known to have been arrested: Carlos Calle, Dr Domi and his wife Maria Dipace, Pedro Landeyro, Antonio Misetich (who worked for a time at MIT's National Magnet Laboratory), Santiago Morazzo, Pedro Victoria. We know a little about their situation because Dr Virgilio Victoria-Troncoso, brother of Maximo Pedro Victoria and an ophthalmologist at the University of Ghent, Belgium, has recently been to see his brother (who has worked overseas, for example at Lawrence Radiation Laboratory) in prison in Argentina. He reports that the scientists, physicians and psychiatrists he saw in the Villa Devoto prison in Buenos Aires were kept in 35 cells, five to a one-bedded cell with dimensions 3m by 2.40m. They have been without meat for months. They have no legal representation, no charges have been brought against them and it is impossible to bring a writ of *habeas corpus*. "My brother", says Dr Troncoso, "has been beaten up and he has lost teeth. The situation is worse than in a novel of Kafka".

It is difficult to see why nuclear scientists have been imprisoned except

that there was a technical difference of opinion some time ago in which Dr Victoria, amongst others, was involved. It concerned the choice of fuel for a new reactor, and Dr Victoria and colleagues favoured natural uranium whereas a group of military scientists favoured enriched uranium. The former view prevailed and it may be that the military, which has in large part taken over leading civilian positions, is determined to settle an old score.

What can scientists do in the face of the widespread breakdown of all the conventional respect for the intellectual life and academic freedom? Amnesty International (53 Theobalds Rd, London WC1) can provide extensive lists of missing Argentinian academics who could be written to—preferably in Spanish, because the authorities are unlikely to pass on anything in English. Dr Victoria's address, for instance, is now 649, Unidad 2, Sierra Chica, Province of Buenos Aires, Argentina. A letter to Excelentísimo General Jorge Rafael Videla, Presidente de la Republica Argentina, Casa Rosada, Buenos Aires, Argentina couldn't do much harm. And maybe it is going to be necessary to try and find academic places outside Argentina for these victims of extremist politics. □

Watershed for poisons

Colin Norman reports from Washington on US efforts to legislate on the controversial matter of toxic substances

A FEW years ago, a group of industrial chemicals called polychlorinated biphenyls (PCBs) were enjoying spectacular commercial success, finding their way into a vast assortment of products ranging from electrical transformers to copying paper and food packaging. In fact, PCBs are so ubiquitous, and so indestructible, that virtually everybody living in an industrialised country now has detectable levels of the chemicals in his or her tissue. They are even turning up in human breast milk, they are present in the flesh of fish from many lakes and rivers, and they have been found in the bodies of animals from such remote places as Greenland. A number of studies have shown that, when fed in relatively large amounts to animals, they may cause cancer, reproductive disorders, metabolic abnormalities, hair loss, skin eruptions, and other health problems.

Use of these versatile substances has now been curtailed in the United States. After five years of trying, the US Congress has finally passed landmark legislation requiring many manufactured chemicals to be screened and tested for toxic effects before being marketed. The grim saga of how PCBs became widespread environmental contaminants long before their health hazards were fully known is, unfortunately, far from unique. Other well-known cases include vinyl chloride, asbestos, bis(chloromethyl) ether, carbon tetrachloride and so on. And, though such occurrences are far from new (one novel theory even suggests that the decline of the Roman Empire may have been caused, in part, by lead poisoning from cooking utensils and wine vessels), they seem to be growing more numerous.

It is not surprising that there are such frequent outbreaks of panic about the toxic effects of widely used chemicals. Civilised man is surrounded by thousands of synthetic chemicals, few of which have been screened for toxic effects, and even fewer have been exhaustively tested. According to an estimate published last year by the Manufacturing Chemists' Association, for example, some 6,500 new chemical products reach the market each year. Yet only about 3,000 chemicals have even been tested for cancer-causing properties, in spite of widely accepted estimates that between 60 and 90% of human cancer is caused by environmental factors.

In short, until now, chemicals have generally been accorded the same legal rights as people—they are assumed innocent until proven guilty. But last week, after years of argument, Congress took a significant step toward writing a new bill of rights for manufactured chemicals. It passed legislation, known as the Toxic Substances Control Act, which gives the Environmental Protection Agency (EPA) broad authority to require pre-market testing of some compounds, and the power to seek a court order to keep potentially hazardous chemicals off the market.

The bill is a landmark piece of legislation for which environmentalists, some trades unions and health scientists have long fought. For the first time, it gives the federal government the power, in theory, to keep an eye on all manufactured chemicals, and it provides at least a coarse filter to screen out potentially troublesome compounds before they do much damage. In the colourful words of Senator Warren Magnuson, a key supporter of the bill, it will "no longer allow the public or the environment to be used as a testing ground for the safety of these products".

Five years of effort

It took five years of intense argument in Congressional committee rooms and behind the scenes to get any toxic substances legislation passed at all. The bill's genesis was a 1971 report by the Council on Environmental Quality, which noted that although some classes of chemicals (such as pesticides, drugs and food additives) are regulated by individual laws, there is no federal authority to control the thousands of other compounds which flood the market each year. A year later, the Senate and the House both passed toxic substances bills, but they were markedly different, and intense lobbying by industry and by the Nixon Administration prevented a final version being passed. The same thing happened in 1973 and 1974, and until very recently it looked as though Congress would again fail to reach agreement on the legislation. The deadlock was broken during what one participant described last week as "three intense bargaining sessions" between Senators and House members.

What emerged was consequently very much a compromise measure, a fact which is reflected in comments last week from spokesmen for groups on

both sides of the fence. "We support the bill as a workable compromise", says Linda Billings, a Sierra Club lobbyist who has been following the legislation throughout its tortuous journey through Congress. She noted, however, that it contains a number of weaknesses and loopholes which will keep many corporate lawyers busy. Similarly, a spokesman for the Manufacturing Chemists' Association (MCA), the industry's lobbying arm, said that the bill, "while tough", is acceptable to the industry. He added, though, "I wouldn't say that we are real happy with everything that's in the bill". Spokesmen for some trades union organisations have also expressed satisfaction that a bill has finally been passed.

One lingering doubt, however, is whether or not President Ford finds the bill to his liking. Throughout the Congressional fight on the legislation, the Administration has lobbied against the measure, arguing that it would be expensive and would constitute too much government regulation of private industry (a theme frequently sounded in Ford's campaign rhetoric). But, since the bill now has broad support, and since a Presidential veto would give Jimmy Carter a gilt-edged campaign issue to exploit, Ford is expected to sign the legislation.

A key feature of the bill—which Ms Billings describes as a "watershed"—is a requirement that EPA must be informed at least 90 days before any new chemical compound is placed on the market, or an existing compound is sold for a new use. (The bill specifically exempts chemicals produced in small amounts for research purposes, it should be noted.) The manufacturer must also send along whatever information he has on the toxicity of the compound, the amount to be manufactured, the likely human exposure, and so on.

Then, if the EPA Administrator decides that the compound is likely to pose a hazard to the environment or to human health, or if he finds that there is insufficient information to judge the hazards, he can issue an order restricting or banning sale of the compound, at least until the required test data is available.

Sticking point

The EPA Administrator is clearly given very flexible authority to determine which compounds should be exhaustively tested, and which should be let through the filter. That aspect of the bill has been the chief sticking point which has held up final agreement in Congress for the past five years. The deadlock was broken by writing into the bill a provision requiring that the Administrator's decisions

must be reviewed by the courts if a manufacturer feels he has been unfairly treated. In short, if a manufacturer objects to an EPA ruling, the agency must seek a court injunction to put the ruling into effect. If EPA doesn't go to court, the ruling would be nullified.

Although that provision may seem like a huge loophole through which smart corporate lawyers can emasculate the legislation, many observers feel that in fact it will be relatively easy for the EPA to obtain an injunction. All that would be required, according to the bill, is a showing that the compound "may present an unreasonable risk of injury to health or the environment", or that it "may reasonably be anticipated to enter the environment in substantial quantities", or that there has been insufficient testing for the hazards to be "reasonably determined or predicted".

Thus, the wording is so general that EPA should have a fairly easy time in proving its case, a fact which made the provision acceptable to environmentalists. "We don't like it", Ms Billings said last week, "but we recognise that it is the best we could get". Similarly, the chemical industry sees some merit in the provision. James Hanes, chief counsel for Dow Chemicals, who has been one of the most outspoken opponents of the bill, told *Nature* that the idea "at least gives the industry the chance to contest the decision out in the open", though he noted that "I don't really see the courts requiring an overwhelming burden of proof here".

If EPA obtains its injunction, it can then specify what tests it requires, and the industry would then be in the position of having to prove that a product is safe before placing it on the market.

Priorities committee

As far as existing chemicals are concerned, the bill requires the setting up of an inter-agency committee charged with the task of drawing up a list of chemicals whose toxicity is open to

question. The committee will assign priorities to chemicals and, within 12 months, the EPA Administrator is required either to issue orders for testing the top 50 compounds, or to explain why he feels testing isn't necessary.

The bill will clearly place a huge burden on EPA, which is required to go through masses of data on thousands of chemicals, decide which need testing and which should be allowed on the market, go to court to obtain injunctions, and exercise considerable judgment on what actions are necessary. Therein lies the bill's greatest potential weakness, according to many observers.

To carry out this Act, the bill authorises expenditures of only \$10 million this year, rising to \$12.6 million next year and \$16 million the year after. Those figures should be compared with the \$125 million which EPA was budgeted to enforce the Clean Air Act, or the \$200 million a year budget of the Food and Drug Administration. It is difficult to see how EPA can effectively enforce the Toxic Substances Act with such small resources, and some observers have suggested that the upshot will be that EPA will be forced to concentrate on a few chemicals and allow many to slip through the net.

Monetary matters have also been a major source of concern to the chemical industry throughout the long Congressional fight over the bill, though for different reasons. There have been numerous studies of what the bill may cost the industry in terms of testing facilities, legal costs and administrative requirements. Large-scale animal tests are very expensive to conduct—according to estimates given in testimony before a House committee by officials from DuPont, for example, a complete battery of tests can cost up to \$500,000. Such figures have thus led to arguments that the bill will stifle the development of important chemicals which may only have a short production run. Some of that concern

has, however, been reduced by the provision exempting research chemicals from the bill.

Assessing the cost

As for the total impact of the bill on the industry, Dow Chemicals has so far come up with the highest figure, estimating that the legislation will cost chemical manufacturers up to \$2,000 million a year. The MCA thought that the cost would probably lie between \$360 million and \$1,300 million, while EPA suggested that it would only be about \$80–140 million.

Whatever the cost may turn out to be, it will be relatively small compared with the massive total sales volume of the industry, or with the costs of treating cancers and other diseases related to environmental factors. The large cost of animal tests, moreover, should provide a strong incentive for industry to develop accurate, short-term *in vitro* tests, such as the system developed by Dr Bruce Ames at the University of California. Already, many large firms are looking closely at such systems, and are using them as rapid screening devices, essentially to develop priorities for animal tests.

It now remains to be seen whether or not President Ford will sign the bill. If he refuses, he not only runs the risk of the matter developing into a campaign issue, he would also probably do the chemical industry more harm than good. This bill swept through the Congress with support from both Democrats and Republicans, there is certainly strong public pressure for such legislation—especially since the panics over PCBs, vinyl chloride and so on—and the polls now indicate that the Democratic majority in the Congress will be much stronger next year. All those considerations suggest that an even tougher bill would emerge from the next Congress if this bill expires. That is one reason why the chemical industry has decided to give this bill at least its lukewarm support. □

USA

Science Court on guard

A number of prominent scientists and government officials met last month to discuss a proposal to establish a 'Science Court' in the United States, to examine complex scientific issues which have a bearing on public policy. The proposal (see box) has recently been receiving considerable publicity in the United States. Wil Lepkowski reports from Leesburg, Virginia

At first blush, the idea of a Science Court sounds a bit intriguing if not a mite exciting: a forum of distinguished scientists and engineers gathered together in court for sifting, filtering, and distilling contradictory facts into one sparkling supernatant of truth. The world of political decision-making is so messy, proponents of the science court assert, that officials would welcome a little plain, unvarnished truth from a

forum that has no political stake in public issues.

This is a fairly accurate, if not precise, description of the idea behind the science court, which is mainly the invention of Dr Arthur Kantrowitz, president of the Avco Research Laboratories. For about a decade, Dr Kantrowitz has been tirelessly giving on and off the cuff speeches on the need to establish such a court and, after a good deal of effort, he finally succeeded in securing government support for a colloquium on the subject. It was held last month at the Xerox Cor-

poration Training Center in Leesburg, Virginia.

About 300 people came, representing law, science, engineering, industrial management, government administration, social science, and general subjects. No one from the humanities was observable, unless one counts anthropologist-oracle Margaret Mead. Dr Mead was the sole bearer of mirth into what was otherwise a dull, juridical gathering. She was in general kind, affirmative, but at one point called the affair a publicity meeting. That may be unkind to Dr Kantrowitz, who in sincerity is outranked by no one. But the whisperings around Washington in that small circle called the science policy community (not to be confused with the working scientists) is to give Kantrowitz his day and let the whole idea collapse under its own unworkable pretensions.

Nothing was really decided at the colloquium. Individuals theorised how it might work, what might be the snags, how the right panels of experts would be picked according to the issue under debate, who would choose the experts, how information would pass between the court and the regulatory agency subscribing to it, what the procedures would be for cross examination, how the public would participate, whether the system already works well enough. In short, the meeting was a *mélange* of speculation. It is probably accurate to say that Dr Kantrowitz, who heads a science court task force set up by a now-disbanded White House science advisory committee, will get a little money to do a science court experiment with a regulatory agency. That's what he intends to do. In fact, the colloquium's point was to hash over the elements of such an experiment.

Two concrete issues were discussed around which an experiment might be devised: food additives and nuclear power. Both issues have enjoyed and lamented over huge outpourings of fact, opinion, and acrimony over many years. In both cases the matter boiled down to the free flow of information—facts withheld by government and industry that no science court could pry loose. And anyway, one could make a fairly substantial case for the "fact" that science courts already exist—through panels serving regulatory agencies, through the National Academy of Sciences, through existing legal courts, in other words through a system within the American democracy that, after all is said and done, does indeed spot the scallawags.

It is certain that the experiment will be tried. The President's science adviser, Guyford Stever, gave it cautious endorsement. Even the Commerce Secretary, Elliot Richardson, said it might as well be tried, although

Science court: specifications

A top-level science advisory committee, established by President Ford last year to prepare the way for the re-establishment of a White House science policy office, recommended that the Science Court idea should be given serious consideration. It established a task force, chaired by Dr Arthur Kantrowitz, which has drawn up the following specifications for the court:

Initiation

1. The experiment will begin with agreements between the Science Court Administration (SCA), the host institution and the funding institution concerning the plans for the experiment.
2. The first step will consist of contacts with regulatory agencies and others to search for a suitable issue. The first issue will be presumed to be bi-polar and will be presumed to be a value laden decision which must be made by the agency and in which the scientific facts are apparently in doubt.
3. An agreement with the regulatory agency to supply necessary legal powers for uncovering non-public information if necessary.

Organisation

4. The SCA, equipped with an issue and funds, will seek Case Managers (CMs) for each side of the issue. The work of the CMs will be funded by the SCA.
5. The SCA will issue procedures, suggested referees, and a panel of prospective judges.
6. The CMs will review these suggestions. The experiment will go forward only after both CMs agree on the referee, the judges and the procedures. A signed agreement to proceed will commit the SCA, the funding agency, the regulatory agency, the CMs, the judges, and the referee.

Suggested procedures

7. The CMs formulate a series of factual statements which they regard as most important to their cases. Such statements must be results or anticipated results of experiments or observations of nature.

The statements should be ranked in order of importance assigned by the CM.

8. The judges examine each statement to determine that it is a relevant scientific fact.

9. The CMs then exchange statements. Each side is invited to accept or challenge each of the opponents statements.

10. The list of statements accepted by both sides will constitute the first output from the SC.

11. Challenged statements are first dealt with by a mediation procedure in which attempts are made to narrow the area of disagreement or to negotiate a revised statement of fact that both CMs can accept.

12. The mediated statements are added to the SC's output. Those statements which remain challenged are then subjected to an adversary procedure.

13. CMs prepare substantiation papers on statements remaining challenged and transmit these to the judges and the opposing CMs starting with the first (most important) challenged statement.

14. The substantiation is cross-examined by opposing CMs and judges and contrary evidence is presented and cross-examined.

15. A second attempt to negotiate a mediated statement is made and if successful this statement is added to the Science Court's output.

16. If this is not successful the judges write their opinions on the contested statement of fact.

17. This procedure is repeated for each of the challenged statements.

18. The accepted statements plus the judges' statements constitute the final output of the procedure.

it shouldn't be confused with a court of law. Stever did imply, however, that one shouldn't expect too much.

The mythology behind the science court concept does deserve an analysis that the colloquium did not consider. The question concerns reasons why the science court idea is deemed so urgent as to have a whole colloquium called to discuss it. Industrialists seem to want it because they are convinced that strident opposition to technology by environmentalists are stifling their own freedom to make fair profits. Scientists and engineers seem to be for it because it gives them the chance to establish order in forums often run emotionally. Environmentalists and public interest figures are against it because they fear their positions will be muted by a scientific elite that has little feeling for human needs. And lawyers are against it because they feel court procedures, if done right, hear all sides to a case, within human limits of patience, tolerance, and intelligence.

On the scope of reality, the science court seems of little importance beyond a possibly interesting intel-

lectual exercise. Regulatory agencies make literally dozens of decisions daily. Hundreds of scientific panels are called to aid in those decisions—little courts, if you will. The National Academy of Sciences runs a series of Science Forums every few weeks or so in which the public actually does debate with scientists the validity of scientific findings relevant to public issues. The administrators of the Science Forum aren't pretentious enough to believe their procedures heavily influence decisions. But some follow-up studies do indicate that the debates raise the personal and collective consciousness to a degree satisfactory to anyone wise enough to understand that people aren't swayed very easily.

The trouble is that no one quite knows what to think of the Science Court. It is a difficult target to attack because the subject is virtuous. Many simply don't feel it is worth much. One might call the feeling the accumulated wisdom of those who have been through the course of using scientific facts in the pursuit of the common weal. □

SWEDEN

Swept in on the green wave?

From Stockholm, Wendy Barnaby assesses what the result of the recent Swedish general election could mean for science in that country

AFTER 44 years in power, Sweden's Social Democrats have been defeated at the polls. Their general approach to science was not discussed in the election campaign. But scientific assessments became very important in the debate which dominated its final stages: whether or not to push ahead with the Social Democrat's plan to make Sweden the world's largest producer of nuclear energy *per capita* by 1990. The scientific input was dramatised by a telegram sent two days before the election by the American Union of Concerned Scientists to the Prime Minister, Olof Palme, challenging his view that nuclear waste disposal problems are on the way to being solved.

Although the non-socialist parties which will now form a coalition government are split on the nuclear issue, the pre-election battle lines were drawn between the Social Democrats on the one hand and the anti-nuclear energy Centre Party, the largest non-socialist party, on the other. And in spite of the enormous organisational and governmental machinery at the Social Democrats' disposal, it was the arguments of the non-establishment scientific community which ultimately appealed to the Swedish voters.

The Centre Party, traditionally the political voice of the Swedish farmer, has in the past few years been the champion of the so-called 'green wave': demands for decentralisation of population and power, environmental protection, the careful use of finite resources and—in the energy field—the development of reliance on renewable resources such as wind and solar power. According to the party's spokesman on scientific affairs, Bengt Sjönell, one of the new government's aims will be to bring the Swedes into balance with nature as far as energy resources are concerned. The first test of how this rather idealistic hope is to be realised will come less than 2 months after the formation of the new government, early in October, when the squat Barsebäck 2 nuclear power reactor, becomes ready for fuel insertion.

The Centre Party's leader (the new Prime Minister), Thorbjörn Fälldin, is personally and publicly committed to stopping the loading of fuel into any more reactors. (He has also promised close down those five reactors already in operation.) Nobody doubts

the strength of his commitment. But the problem with Barsebäck 2 is how fuel loading can be prevented. One proposal is to prohibit the insertion of fuel by passing a succession of laws each valid for one month at a time until compensation can be negotiated with the reactor's owners, who have demanded \$445 million from the government. The trouble with this is that the reactor is owned by a private company, Sydkraft, and for a non-socialist government to make its legislative debut with such regulation of private industry would be politically unpalatable. To get around this difficulty, it has been suggested the Nuclear Power Inspectorate—a state body which checks reactors under construction and in operation to see that they conform to agreed standards—could prohibit fuel loading at Barsebäck 2. But that organisation is itself not at all happy with the idea. Its brief covers purely technical areas, and although the checklist for the reactor has not yet been entirely worked through, there have so far been no technical hitches. Dr Arne Hedgran, a representative of the inspectorate recently made it quite clear that the organisation does not want to become involved in the government's political problems.

The tangle over Barsebäck 2 is the most pressing problem faced by the new government; not only because of the time factor but also because the Centre Party's credibility will be greatly affected by its handling of the situation. In its very first days of power it will have to come to grips with the issue held to be largely responsible for its victory.

The influence of the already-powerful environmentalist groups will no doubt increase under the new government, partly because of official willingness to listen, but also because of significant gains by the environmentalists in the local elections held at the same time as the national ones. One such local result has particularly far-reaching national and international implications. A victory by groups opposed to the mining of uranium near Billingen, 350 km south-west of Stockholm, means that uranium reserves estimated to be the West's largest in the next economical price range cannot now be exploited for at least three years. The mining firm LKAB had been hoping to mine them either for domestic use or for export.

Beyond the immediate problems, what sort of initiatives in science policy can be expected from the Centre Party? It should be mentioned that Sweden



Fälldin, committed

has no ministry of science: the administration of science instead falls to various other ministries. Those most concerned are Education, Industry and Agriculture, but even they exert little pressure on the direction of research. Once funds have been allocated to the extraordinarily varied array of institutes and research bodies, each research group is practically autonomous in deciding how they are to be spent. Consequently, it is often hard to see whether Sweden has any science policy as such at all.

One trend which is, however, apparent in all this diversity is the priority given to applied research over basic research. This emphasis has been responsible for the present plight of so-called basic technical research, which has been practically squeezed out of existence in Sweden. Bengt Sjönell recently declared that the Centre Party wants to strengthen the relative position of basic research, and stressed specifically that support for basic technical research would be increased. But the party's intentions have not yet been expressed in detailed proposals, or even in concrete plans, though in tertiary scientific education, the party will try to tempt students back to research in the natural sciences, whose current unpopularity has been raising questions about Sweden's long-term scientific research capabilities. But Sjönell seems unaware of the potential of the International Energy Institute due to begin work here next year. Although the government will have no control over the work programme at the institute, it is a reasonable bet that much of the research done there will be directly relevant to official aims. And for a government whose interest in science is concentrated in the energy field, the value of such research should not be underrated. In other areas of Swedish science, the prognosis is business as usual. □

IN BRIEF

NIH budget veto overridden

A few days before it adjourned for the November elections, the US Congress firmly overrode a Presidential veto of a budget bill for the National Institutes of Health (NIH), thereby increasing NIH's biomedical research funds by more than \$200 million in the fiscal year which began on October 1. The bulk of the increase will go to the National Cancer Institute (NCI), whose budget will soar from \$762 million to \$815 million. Also set for a large increase is the National Heart and Lung Institute, which will get \$397 million, up from \$370 million last year. The final budget levels represent a considerable victory for supporters of the cancer programme, for the Ford Administration had recommended that the NCI's budget be held approximately

constant and that the other NIH institutes be given modest increases.

Air Act problem

The US Congress has handed the automobile industry a tangled legal problem by failing to pass amendments to the Clean Air Act before it adjourned last week. The amendments would have relaxed emission control standards for 1978 model cars, which the industry is already gearing up to produce. Members of Congress failed to agree on the bill, and it died when the session ended in the early hours of Saturday morning. Thus, unless the new Congress approved laxer standards when it convenes next January, the industry will be faced with the choice of producing illegal cars or closing down. That, in fact, is exactly the situation that the

industry has been trying to engineer. It believes that the threat of wholesale closures and high unemployment in Detroit will be sufficient to force the new Congress to meet its various demands.

UK changes

Under the rearranged provisions for science policy formulation in Britain, the UK government has appointed a 37-year-old biochemist from Essex University, Professor John Ashworth, to the Cabinet Office's Central Policy Review Staff for a two-year period.

Elsewhere, Professor J. L. Gowans, 52, will be the next Secretary of the UK Medical Research Council, taking over from Sir John Gray in April 1977. He is presently Director of the MRC's Cellular Immunology Unit.

ALTHOUGH the sixth report of the Royal Commission on Environmental Pollution, and various informed comments on it, have shown that there is widespread concern in Britain about the rapid development of nuclear power in general, and of the fast breeder reactor in particular, those responsible for the safety of atomic energy and nuclear fuels have come well out of the controversy. Engineers, scientists and those working in nuclear power stations have behaved responsibly in this respect, and have scrupulously observed the most rigorous safety precautions. Many of us may doubt whether we need this massive development in electrical generation which the Department of Energy thinks necessary, when we already have 50% more capacity than the peak load, and when demand is falling, but that is another matter. There is little doubt that if the nuclear generating industry is expanded as planned (*sic*) the same high standards of care will continue to be exercised, though the statistical chances of accidents must inevitably increase.

Therefore if we are to have more nuclear power stations, we must constantly review and improve safety precautions. At present there is a series of safety regulations laid down and agreed internationally; unfortunately some of these are not entirely practical. Thus the specifications for the containers, each weighing some 45 tonnes, in which nuclear fuel is shipped around the country, are intended to ensure that the risks of an escape of radiation or radioactive materials are very small indeed. The vessels are designed to resist such mishaps as a fall of several metres, though there is doubt whether they

are strong enough to remain intact if the train carrying them was derailed at a high speed.

The regulations state that they must be able to resist a temperature of 800 °C, uniformly applied to the

Protection practice

KENNETH MELLANBY

whole of their surface. Unfortunately the experts of British Nuclear Fuels Ltd have found it impossible to devise a technique by which the containers can be subjected to the conditions laid down by the international authorities. They have spent—or wasted—some half a million pounds trying to devise a method of giving exactly the correct exposure to this high temperature. As this sort of temperature exposure appears to be impossible to produce, it is difficult to understand why this particular standard was set. We wish to be sure that our nuclear fuel is safe when the containers are

exposed to the sort of hazard which, in practice, is likely to be encountered. Uniform conditions of 800 °C continued for twenty minutes do not constitute one of these. It would surely be better to devise a "standard fire" similar to one likely to be experienced by the nuclear fuel when in transit, and to put the containers into it.

A similar attitude to that which sets up these unreal safety standards may sometimes be demonstrated by those responsible for our defence. Some years ago conservationists were urging the War Office to give up an area of Britain's coastline, where the sand dunes were of great natural history interest. The War Office was reluctant to part with the land, giving as an excuse that it was of the greatest importance for military training because it was "unique" and irreplaceable by any other area which was available. When I heard this I could not help wondering how it was to be used for training our forces. If it was indeed unique, then any military exercise performed there would have little relevance to those done on other, quite different, terrains. In fact the only value of this special piece of coastline would be in order to train our forces to defend it. Surely training would be better done on a more typical area, with conditions similar to those found in many parts of the globe?

We need to be protected from danger by safety standards for hazardous industrial processes, and by the possession of efficient military defences. In both cases we should first decide exactly what we need to be protected from, and then we can set up practicable standards to which we can work.

news and views

Doping amorphous silicon

from Andrew Holmes-Siedle

Recently (see *News and Views*, **260**, 667; 1976) a group at the University of Dundee (Spear *et al.*, *Appl. Phys. Lett.*, **28**, 105; 1976) reported that, for the first time, a p-n structure had been made from amorphous silicon only. This achievement demonstrates that, contrary to common belief, amorphous material can be "doped" by impurities. Such a finding effectively eliminates one of the hypotheses which has been aired to explain the suppression of doping in amorphous materials—namely that the valence bond of the dopant atom (which cannot be satisfied within a constrained crystal structure) can usually be accommodated and satisfied by an atom of the disordered matrix. Here, however, is a truly amorphous silicon matrix which can be made either p-type or n-type by the addition of boron or phosphorus, although more dopant is needed than for the case of crystals.

Spear and LeComber have now

published some more basic investigations on conductivity and field effects in a much simpler structure, namely a silicon film of only one conduction type (Spear and LeComber, *Phil. Mag.*, **33**, (6) 935-949; 1976) and their new account shows that my earlier comments on the "apparent inefficiency of doping" and my implication that disorder in these silicon networks was still largely "negating" the electrical activity of the dopant atoms was an oversimplification. In fact, quite a large fraction of the dopant atoms is ionised, as we shall see below.

Spear and LeComber deposit the silicon films by glow-discharge decomposition of silane on glass, using phosphine or diborane as dopant gases. This is a well-known technique but is the first intensive study of the product. They found that the conductivity of the films could be controlled in unprecedented fashion for amorphous materials, from 10^{-7} to 10^{-2} (ohm cm) $^{-1}$.

In the past, sputtered films have usually had very low conductivity and any changes achieved (for example by heat treatment—see stippled area of figure) were caused by changes in the density of intrinsic defects, sometimes called "dangling bonds", rather than by true doping effects. Such ability to control conductivity could open up the technological uses of thin-film amorphous silicon—a very desirable thing because of the cheapness and large possible areas of such films. However, there are some formidable, fundamental and technological barriers to be surmounted before devices competitive with single-crystal silicon forms can be created. The efficiency of doping as it affects conductivity is still quite poor, for some fundamental reasons. First, the mobility of carriers in the disordered matrix is lower than for single-crystal semiconductors. Second, many carriers are immobilised in a class of energy states special to amorphous semiconductors ("mid-gap states"). The achievement of Spear and LeComber is to reduce these states until at least a small proportion of the electrons and holes, freed from the dopant atoms, appear in conduction states. Nevertheless, the poor relative efficiency of dopant atoms in producing conductivity is clearly shown up in the figure, adapted from the new measurements of Spear and LeComber (crosses) and other data (full lines, dotted lines). N_D^+ was estimated tentatively by Spear and LeComber from the temperature dependence of conductivity in the amorphous films, with supporting evidence from chemical analysis and photoconductivity measurements. The shaded area shows the difference between amorphous and single-crystal material. While further work may bring these curves somewhat closer together, there is no immediate clue as to how this may be done. In the new material, the density of electron states

A FOOTNOTE to the work reported here is now required because, as might have been expected, the possible use of amorphous silicon films to make photovoltaic p-n diodes has not been ignored. The first to publish is RCA Corporation; others will probably follow shortly. The RCA workers, D. E. Carlson and C. R. Wronski (*Appl. Phys. Lett.*, **28**, 671; 1976), have made thin-film, back-surface amorphous-silicon solar cells on glass by a deposition process essentially the same as Spear and co-workers at Dundee (glow-discharge deposition of silane gas) but the RCA films used are thicker than the Dundee films and have an undoped ("intrinsic") region of thickness about $1.5\ \mu\text{m}$ between the p and n-type regions. The $3.5\ \text{cm}^2$ cells produced have a terrestrial solar conversion efficiency of 2.4% and the authors estimate that the theoretical

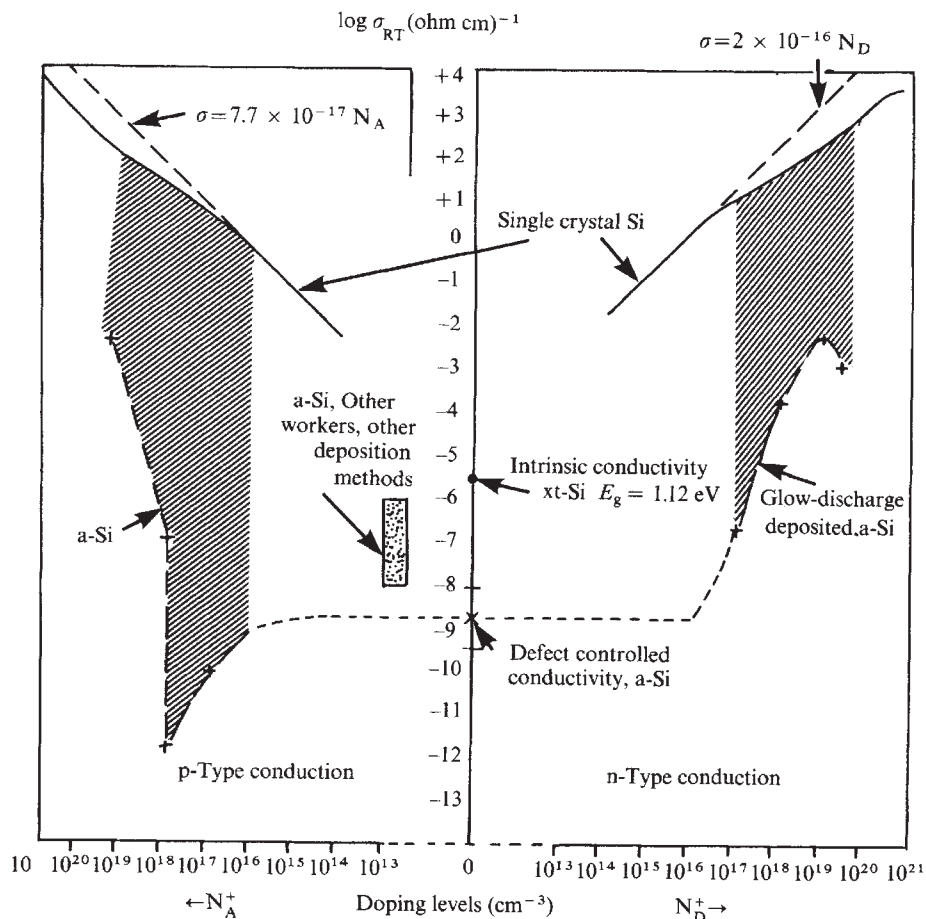
efficiency of their p-i-n structure should be 14–15%. Thus, the unexpected finding that useful junction barriers can be rather easily produced in amorphous silicon may lead to a surge of effort to produce low-cost, large-area solar energy converters by this method. The economic advantages of thin-film devices over ingrown semiconductor devices have been stressed and we may now be avoid the problem of the polycrystalline thin-film approach.

Though surprising advances have recently been reported in fabrication of polycrystalline silicon cells (5% efficiency claimed for large-area polycrystalline films deposited on graphite, *Electronics*, **33**, June 24, 1976) the grain boundaries, which constitute a completely unwanted barrier to carrier transport in such films, are dispensed with in amorphous layers.

in the middle of the gap may be as low as $10^{17} \text{ cm}^{-3} \text{ eV}^{-1}$. This is a huge improvement over previous films, which had densities as high as $10^{20} \text{ cm}^{-3} \text{ eV}^{-1}$ at mid gap, but how much further can we go towards the case of single-crystal silicon, with a density of virtually zero? Some opinion says that the gap in the figure probably cannot be closed much more, but it is still a fascinating question.

Many other fascinating questions will doubtless be raised during further basic physical studies on the new material. With diodes, for example, recombination in amorphous networks can be explored, recombination being the property which most affects gain in transistors. With the doping techniques, we can refine our models of electron and hole transport in regimes not possible before and thereby redress our present ignorance about the environment of substitutional impurities in disordered networks. Out of such work could grow the expertise necessary to exploit this second generation of amorphous films.

Comparison of conductivity against doping curves for single crystal silicon and amorphous silicon (a-Si).



Chlorophyll dimer and triplet states – key roles in photosynthesis?

from Godfrey Beddard

It has been known for some years that the initial photochemical act in photosynthesis is the formation of an oxidant P^+ , and a reductant X^- resulting from excitation of the reaction centre PX, either directly by light absorption or indirectly by energy transfer from accessory pigments. This reaction centre is composed of a special chlorophyll complex, the electron donor, closely associated with an electron acceptor.

The nature of the reaction centres has been the subject of much interest and speculation and recently experiments using optical spectroscopy and magnetic resonance techniques have resulted in a detailed picture of the bacterial reaction centre P_{870} and the characterisation of the two reaction centres P_{700} and P_{680} of green plants. Chlorophyll dimers have been implicated in all three types and recently electron spin resonance (ESR) spectra exhibiting the CIDEP phenomenon suggest that chlorophyll triplet states are also involved, as described McIntosh and Bolton (*Nature*, **263**, 443; 1976).

Picosecond spectroscopy on bacterial reaction centres has revealed transient

absorption changes, implicating both bacteriochlorophyll (BChl), and bacteriopheophytin (BPh). The transient forms in 8 ps and decays with a 150 to 250 ps lifetime concomitant with $P_{870}^+ X^-$ formation. (Rockley, *et al. Proc. natn. Acad. Sci. U.S.A.*, **72**, 2251; 1975; Kaufmann, *et al. Science*, **188**, 1301; 1975). Fajer *et al. (Proc. natn. Acad. Sci. U.S.A.*, **72**, 4956; 1976) proposed that the primary electron acceptor was not an iron-ubiquinone complex as was previously thought, but was instead bacteriopheophytin. In an elegant experiment Fajer *et al.* reproduced the transient absorption spectrum of the reaction centre from the difference spectrum; $(BChl)_2^+ - (BChl)_2$ plus $BPh^- - BPh$. Agreement was found for all absorption bands from 350 to 1,250 nm as a transient absorption of $(BChl)_2^+$ has been detected at 1,250 nm. (Dutton *et al., FEBS Lett.*, **60**, 275; 1975).

Triplet state zero-field parameters have enabled Clark *et al. (Biophys. Biochem. Res. Commun.*, **71**, 671; 1976) to determine that the two BChl molecules *in vivo* are tilted at 48° and rotated by 78° to one another. Further-

more ENDOR linewidths indicate dimeric BChl species are present, (Feher, *et al., Ann. N.Y. Acad. Sci.*, **244**, 239; 1975; Norris, *et al., ibid.*, 239; 1975) and in addition electron spin polarisation indicates that the primary act is a radical pair formation. (Thurnauer, *et al., Proc. natn. Acad. Sci. U.S.A.*, **72**, 3270; 1975). These experiments support a model of the bacterial reaction (in *Rhodospseudomonas sphaeroides*) in which the charge separation process forms the ion pair $(BChl)_2^+ BPh^-$ before the reduction of the ubiquinone "primary" acceptor.

The chlorophyll-*a* species of photosystem I reaction centres— P_{700} —has a bleachable absorbance at 703 nm and a characteristic ESR signal. *In vivo* ENDOR linewidths indicate that P_{700} contains a $(Chl)_2$ species (Feher, *et al., op. cit.*; Norris, *et al., op. cit.*), and Shipman has recently proposed that this dimer has the form $(Chl-ROH)_2$; with $R=H$, ethyl or protein. Shipman, *et al., Proc. natn. Acad. Sci. U.S.A.*, **73**, 1791; 1976). This symmetrical dimer has absorbance and ESR spectra similar to P_{700}^+ . The oxygen in ROH bonds to the Mg of Chl and the

hydrogen bonds to ring V carbonyl of the other Chl. As ROH, RNH, RSH compounds also form dimers this presents the intriguing idea that the *in vivo* dimer may be bound to similar groups in a protein but at present no experiments have been reported to test this idea.

Perhaps the most interesting observation on P_{700} is a transient electron proton resonance (EPR) emission spectrum produced by a single light flash on chloroplasts at room temperature. (Blankenship, *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 4943; 1975). The EPR signal is thought to be that of the primary acceptor as it does not correspond to P_{700}^+ or a ferredoxin.

The emission is caused by the two spin states, normally thermally populated to 0.1% of one another, obtaining a significant population difference as a result of a particular chemical reaction favouring one state over the other. This phenomenon is called chemically induced dynamic electron polarisation (CIDEP). Immediately after the radical anion of the acceptor is formed the spin states still have the same populations as the anion's precursor and a CIDEP signal is observed until spin lattice relaxation equalises the spin populations. A triplet state of Chl is thought to be responsible for the emission from photosystem I, as a radical pair, which can also generate CIDEP are unlikely to diffuse together in the reaction centre.

There is still much controversy about the identity of the primary acceptor for P_{700} . Malkin *et al.* (*Biochim. biophys. Acta*, **430**, 399; 1976) maintain that the acceptor is a ferredoxin, but McIntosh and Bolton (*Biochim. biophys. Acta*, **430**, 555; 1976) and Evans *et al.* (*Nature*, **256**, 668; 1975) dispute this and claim that a species with ESR signal $g=1.76$ is the primary acceptor and that ferredoxin has a secondary role. The relationship of the signal seen by Blankenship to these acceptor signals is not known. The evidence is not as complete as with the bacterial reaction centre but the initial charge separation in photosystem I appears to produce a $(\text{Chl})_2^+$ species and an unidentified radical anion before the ferredoxin is reduced.

Much less is known of photosystem II reaction centres, P_{680} , than of P_{700} , although there is good evidence that P_{680} is the primary donor, possibly a chlorophyll dimer (Pulles, *et al.*, *Biochim. biophys. Acta*, **440**, 98; 1976). The observation, by McIntosh and Bolton, of a CIDEP emission from Chl in photosystem II is thus of particular interest as this signal can only arise from a Chl triplet state at the temperatures used. They suggest that the triplet state forms very rapidly from the singlet by the formation of a Chl^+

species. The primary acceptor would then have to be adjacent to the dimer in order to accept the electron at a rate much faster than the charge recombination for efficient charge separation to occur.

Many similarities between reaction centres are now emerging. All involve chlorophyll dimers; at least two, and perhaps all, contain short lived "transient" acceptors between the dimer and the "primary" acceptor which is perhaps a device to prevent rapid charge recombination of the ion pair. Although both P_{700} and P_{680} appear to involve triplet states formed, perhaps very quickly, after excitation of the dimer species the reasons for preferring triplets over singlets are unknown. Consequently picosecond spectroscopy will be invaluable in observing transients produced in the initial processes and our present knowledge of reaction centres should provide impetus to produce model systems to mimic photosynthetic processes. $\square$

Homostable operons?

from E. G. Richards

READERS who glance through the pages of the more theoretical journals will be aware of the immense activity over the past 15 years on the statistical mechanical basis of the melting of DNA. Certainly every well-informed undergraduate will know that DNA undergoes a cooperative helix coil transition when heated that gives rise to a sharp melting curve. However theory suggests that things are not quite that simple. For instance, a hypothetical DNA molecule consisting of a region of 1,000 A-T base pairs followed by a similar one of G-C pairs would give a melting curve consisting of two sharp steps. To produce a sharp step, theory suggests, requires a 'homostable' region of 500 or more base pairs with no sequences of more than a few adjacent A-T or adjacent G-C base pairs. A DNA molecule consisting of several such homostable regions would then melt in a series of sharp steps, and the melting temperature of each step should reflect the base composition of the corresponding region.

Another and more informative way of depicting melting behaviour is by the differentiated melting curve in which sharp steps now appear as sharp peaks. In recent years much expertise and ingenuity has been expended in constructing equipment capable of producing such curves at high resolution so that the fine structure of a melting curve occurring over 10 °C or so could be resolved into a dozen or more sharp peaks each a degree or so in width.

Wada and Tachibana, in a paper in *Nature* (**263**, 439; 1976) note that short DNA species do indeed manifest such fine structure in their differentiated melting curves and, moreover, that the melting temperature of the individual peaks does indeed bear the expected relation to the base composition of the corresponding DNA region as revealed by spectral analysis. In longer DNA species, presumably consisting of many homostable regions with closely similar stabilities, the fine structure is less apparent.

These authors then go on to consider the behaviour in more detail of a short DNA (fd phage, 6,000 base pairs) and fragments produced from it by the restriction enzyme *RH*inHI.

First, the intact DNA (RF linear) is shown to have a differentiated melting curve consisting of more than a dozen sharp peaks distributed over a 10 °C melting range. The product formed by the action of the restriction enzyme and consisting of three fragments gives a broadly similar curve. When the three fragments were separated by polyacrylamide gel electrophoresis they individually gave curves with considerably fewer peaks. Nevertheless, when the three curves corresponding to the three fragments were summed, the result was closely similar to the curve of the mixture.

These results suggest that the peaks in the differentiated melting curves arise from homostable regions situated at specific locations in the molecule. It would appear from the relative sizes of the peaks and from the fact that splitting is observed that some peaks correspond to several homostable regions with closely similar stabilities.

A detailed comparison of the curves for the intact molecule and for the mixture of its fragments suggests that one homostable region may affect the melting temperature of others so that peaks in one of the curves may be split in the other by moving to slightly different temperatures. It is tempting to speculate that such interactions are between adjacent homostable regions, in which case it might be possible to map the regions along the molecule. Certainly the effect presents interesting statistical mechanical problems.

Wada and Tachibana end by speculating as to whether these homostable regions have some biological role. The data suggest that each operon can contain only a few and perhaps only one homostable region, and the authors suggest that the regions might play some part in recombination. They also note that wobble interactions in translation could well mean that homostable regions and active polypeptide sequences could be selected for simultaneously. $\square$

Photosynthetic prokaryotes

from Venetia A. Saunders

The second international symposium on photosynthetic prokaryotes and first meeting of the Federation of European Microbiological Societies was held at the University of Dundee, Scotland, on August 22–28, 1976. It was organised by G. A. Codd, and W. D. P. Stewart of the Department of Biological Sciences, University of Dundee.

MUCH interest in the photosynthetic prokaryotes derives from their evolutionary and ecological significance. Moreover their metabolic versatility makes them particularly attractive research tools for studying processes involved in photosynthesis and nitrogen fixation. It is customary to recognise two groups of photosynthetic prokaryotes, the photosynthetic bacteria and the cyanobacteria (blue-green algae). The cyanobacteria typically exhibit oxygenic photosynthesis with water as the ultimate electron donor. On the other hand photosynthetic bacteria do not evolve oxygen, but utilise oxidisable substrates besides water (notably reduced sulphur compounds or organic compounds) as electron donors in photosynthesis. Now it seems that certain blue-green algae can grow at the expense of reduced sulphur compounds. Are these compounds also utilised by blue-green algae as electron donors in photosynthesis? R. Castenholz (University of Oregon) reported that sulphide could act as a photoreductant in certain blue-green algae from hot springs, but only when photosystem II was switched off. However it is not yet clear whether this process can be used to support growth. H. Utkilen (University of Oslo) demonstrated oxidation of thiosulphate by the blue-green alga *Anacystis nidulans*. He proposed that when the supply of ATP or of electrons from water is curtailed by low light intensity, *A. nidulans* utilises thiosulphate as an electron donor in a manner analogous to that of photosynthetic bacteria.

The primary photochemical reaction in bacterial photosynthesis involves transfer of an electron from bacteriochlorophyll to the primary acceptor. In the purple non-sulphur bacterium *Rhodospseudomonas sphaeroides* this occurs in a pigment-protein complex, the reaction centre. Recently intermediate short-lived states of this primary event have been identified. Interpretation of the nature of such states has been aided by picosecond

kinetic measurements. R. Cogdell (University of Glasgow) and colleagues propose that both bacteriochlorophyll (magnesium porphyrin) and bacteriopheophytin (hydrogen porphyrin) have a role in the primary photochemistry (see *Proc. natn. Acad. Sci. U.S.A.*, **72**, 2251; 1975). F. Reiss-Husson (Gif-sur-Yvette) described a procedure for incorporating reaction centres from *R. sphaeroides* into lecithin liposomes. Such an *in vitro* system should be convenient for studying the contributions of various electron transport components to the reconstitution of photosynthetic activity.

In photosynthetic bacteria the light-harvesting pigments are either accommodated with the energy conversion system on a topologically complex cytoplasmic membrane, or located in different structures of non-unit membranes called chlorobium vesicles. R. C. Fuller and C. Boyce (University of Massachusetts) reported that in the green bacteria reaction centre, bacteriochlorophyll *a* but not the bulk light-harvesting (antenna) chlorophyll is associated with the cytoplasmic membrane. The antenna chlorophyll is restricted to the chlorobium vesicles, which are considered to press up against, but remain distinct from, the cytoplasmic membrane. Light energy is transmitted from these vesicles to the reaction centre. *Chloroflexus* the only known organism with chlorobium vesicles capable of growing aerobically in the dark as well as anaerobically in the light, should accordingly prove an attractive system for investigating the development of this type of photosynthetic apparatus.

Nitrogen fixation has been more extensively studied in the cyanobacteria than in the photosynthetic bacteria. Furthermore the ability of cyanobacteria to fix nitrogen may explain symbiotic associations of certain blue-green algae in lichens and liverworts. Morphological and physiological modifications of the blue-green algae accompany these associations as reported by W. D. P. Stewart and colleagues (Dundee University). Significantly such symbiotic blue-green algae fix nitrogen more efficiently than in their free living state. The heterocyst is the main site of nitrogen fixation in certain filamentous (heterocystous) blue-green algae. Neither CO₂ fixation nor photosynthetic oxygen evolution occurs in heterocysts, such activities being restricted to vegetative cells. E. Tel-Or (University of Dundee) proposed that the defects in the photosynthetic machinery of heterocysts were probably due to the low level of ribulose diphosphate carboxylase, the cardinal enzyme in CO₂ fixation, and depletion of manganese required for

the water splitting reaction of photosynthesis.

Heterocysts develop when blue-green algae are grown in the absence of combined nitrogen. A definite heterocyst pattern emerges throughout the algal filaments. The development of such patterns may well be dependent not only on intracellular but also intercellular reactions. J. C. Meeks (Michigan State University) suggested that some diffusible product of mature heterocysts, possibly glutamine or a derivative, may inhibit nearby cells from differentiating into heterocysts. However the heterocyst pattern is established before nitrogen fixation occurs. What then triggers the developmental events? Studies on the mechanisms of induction of enzymes pertinent to the process of differentiation, as pointed out by R. Haselkorn and N. Woods (University of Chicago), may further elucidate this problem.

The promise of the genetics and molecular biology of photosynthetic prokaryotes is gradually being fulfilled. A novel gene transfer system has been discovered in *Rhodospseudomonas capsulata* by Marrs, (see *Proc. natn. Acad. Sci. U.S.A.*, **71**, 971; 1974). Furthermore this system has been used for mapping genes concerned with photopigment synthesis (B. Marrs, St. Louis University) and also for manipulating genes for nitrogen fixation (J. D. Wall and H. Gest, Indiana University) in this organism. The ability to manipulate genes in photosynthetic prokaryotes will lead to a more profound understanding of the control of such processes as photosynthesis and nitrogen fixation.

In discussing the evolution of the cyanobacterial genome, M. Herdman (University of Dundee) reported a concomitant increase in genome size with increasing morphological and biochemical complexity for these organisms. He speculated from the results that genomes of the cyanobacteria could have formed by multiplication and divergent evolution of copies of a small ancestral genome.

R. P. Ambler (Edinburgh University) reporting sequencing studies on *c*-cytochromes of eukaryotic and prokaryotic photosynthetic systems, urged caution in the interpretation of phylogenetic relationships based on structures of existing proteins. Indeed differences and similarities between species may well have been blurred by the agency of genetic exchange.

A fitting finale to the meeting was the fascinating report by R. A. Lewin (University of California, La Jolla) of prokaryotic "green algae/bacteria". These unicellular algae, found in association with ascidians, possess a unique combination of characteristics, predominantly prokaryotic, but the organ-

isms contain both chlorophyll *a* and *b* as found in eukaryotic plant cells (see *Nature*, **261**, 697; 1976). Could these organisms or relatives be ancestral forms of photosynthetic plastids of eukaryotic cells? Whether this type of "prokaryote" is a missing link or not remains to be seen, but it is likely to be a link between this and the third symposium. □

Anti-juvenile hormone and pest control

from a Correspondent

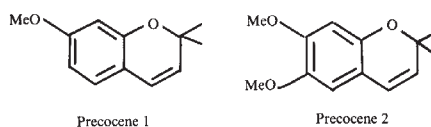
THE preparation of active extracts of juvenile hormone from the American cecropia silkworm 20 years ago suggested the possibility of the eventual use of synthetic hormone as an insecticide. The original idea was that this could lead to the appearance of non-viable intermediates between larvae and adults. The discovery, 10 years later, of the chemical nature of juvenile hormones (of which three varieties have so far been described) stimulated an immense amount of synthetic chemistry in this field. A vast range of products has been produced, some of them not at all closely related chemically to the natural hormone; some, far more active than the natural substance, have been put on the market as insecticides.

The obvious theoretical objection to the juvenile hormone as an insecticide—that it will tend to produce giant larvae which may be more destructive than the ordinary sort—has proved justified in some cases. On the other hand, it has been proved empirically that some of these products are effective for rather special purposes, and some of them have a sterilising action on the adult female or disrupt the development in the resulting eggs. By and large, however, the results have been somewhat disappointing.

It was suggested early that an anti-juvenile hormone, which could cause precocious metamorphosis, might be a more successful kind of insecticide. In a recent paper William S. Bowers and his colleagues at Geneva, New York (*Science*, **193**, 542–547; 1976) report a search for such products in a long series of plants, by extracting the less polar organochemical components in a mixture of ether and acetone. From the common bedding out plant *Ageratum houstonianum* they obtained a product which had very active anti-juvenile hormone properties when applied to immature Hemiptera. It in-

duced precocious metamorphosis at the ensuing moult when applied to first, second or third-stage larvae of the milkweed bug *Oncopeltus*; and similar results were obtained in the cotton stainer *Dysdercus*.

The active principle was purified, analysed and synthesised. It contained two simple chromenes, 7-methoxy-2,2-dimethyl chromene and 6,7-dimethoxy-2,2-dimethyl chromene (Fig. 1), which were already known from plant extracts. On account of their ability to cause precocious metamorphosis these substances have been named precocene 1 and 2. Precocene 2 is about ten times more active than precocene 1.



Eggs of Hemiptera fumigated with the precocenes gave rise to larvae which developed normally for two instars and then moulted to precocious adults. The precocious adults, however produced, had well formed ovaries but they never matured to form ripe eggs—a process for which juvenile hormone is necessary. Application to adult females caused regression of the ovaries; and late treatment had an ovidical action, the nature of which is unknown.



A hundred years ago

WE learn from the *Chronique de l'Acclimation*, that in the just completed New York Aquarium immense basins have been constructed for the reception of the large cetaceans. A number of Otaries have already been received from Behring Strait, and the proprietors hope to be able to exhibit to the public the famous seal Ben Butler, which has for many years frequented the island of San Domingo, in the Bay of San Francisco; the director has offered 5,000 dollars for this curiosity. For the purpose of facilitating scientific researches, the central building contains a library of the best works in natural history, pictures, scientific journals, a laboratory, microscopes, drawing-tables, dissection-room, and all the necessary materials for modelling and photography. Finally, the establishment contains a restaurant in which will be served fish and crustaceans caught before the eyes of the customer. From *Nature*, **14**, October 5, 516; 1876.

The Colorado potato beetle *Leptinotarsa*, on the approach of winter, ceases to secrete juvenile hormone. It enters reproductive diapause, egg development is suppressed and it burrows into the soil. When the adult *Leptinotarsa* was treated with precocene it was forced, apparently permanently, into diapause. The mode of action of the precocenes is not yet known; there are many steps in the synthesis, physiological action or breakdown of the juvenile hormone, any of which could be responsible for the disruptive effects observed.

It remains to be seen whether materials of this kind will prove effective for practical control. The fact that all their effects will diminish insect damage, and that all the precocious females are sterile, are favourable factors. The substances obtained so far have induced precocious metamorphosis only in Hemiptera, and not in Holometabola. But they have sterilised adult females among Diptera and Coleoptera. The discovery of anti-juvenile hormones specific for selected pest species is an attractive possibility. □

Specific toxin receptors in plant disease

from I. M. Smith

THE fungi which parasitise plants are commonly host-specific; one pathogenic species will often only infect a limited range of closely related plant species or even only some genotypes within a species. The molecular basis of this specificity is one of the outstanding problems in physiological plant pathology. Some progress has been made in the type of disease in which successful infection depends on the release by the fungus of phytotoxic metabolites which may be host specific. One notable example of this is the Victoria blight of oats (caused by *Helminthosporium victoriae*), in which the toxin victorin has high specific activity only on certain oat genotypes, which are at the same time the only ones susceptible to infection. Similarly, *H. sacchari* produces the toxin helminthosporoside which is only toxic to susceptible lines of sugarcane. In this case, Strobel has reported a membrane protein which binds the toxin, but only in susceptible lines. Resistant lines have a slightly different protein which does not bind. The immediate consequences of toxin binding are not clear, but this work has been till now the only reported case of a specific receptor for a phyto-

toxin involved in pathogenesis. Now, Steele *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **73**, 2245; 1976) report the specific binding of another phytotoxin (tentoxin from *Alternaria tenuis*) to a receptor protein in susceptible hosts.

A. tenuis is a widespread weak parasite which causes near-saprophytic infections of grain, fruit and senescent tissues generally. It does, however, cause a very distinctive seedling blight of some species, in which the cotyledons become severely chlorotic and the fungus invades the damaged seedling. This chlorosis can be exactly reproduced by treatment with tentoxin, a cyclic tetrapeptide, and it seems that the fungus is dependent on the action of the toxin for successful infection.

Steele *et al.* have now shown that tentoxin shows specificity in three respects. When added to isolated chloroplasts of a toxin-sensitive species such as lettuce, it inhibits coupled electron transport. When added to purified coupling factor 1 (CF₁, a chloroplast ATPase directly involved in photophosphorylation), tentoxin inhibits the ATPase activity. If synthetic tritiated tentoxin is added to CF₁ in a continuous ultrafiltration system, the protein can be shown to have approximately one binding site per molecule for tentoxin, with an affinity constant of $2 \times 10^8 \text{ M}^{-1}$. Comparable tests on an insensitive species (radish) showed only slight inhibition of photophosphorylation, no inhibition of ATPase activity, and an affinity constant less than 10^4 M^{-1} . Other sensitive and insensitive species fitted the same pattern with respect to inhibition of photophosphorylation. It would seem, therefore, that the CF₁ of sensitive species acts as a tentoxin receptor and that toxin-binding leads to inhibition of photophosphorylation. The CF₁ of sensitive species binds poorly and is not inhibited. These results are of great significance, first because of the specificity of the inhibition and binding, and second because the binding may in itself explain the mechanism of toxicity of tentoxin.

There are, however, certain aspects that remain to be analysed. The relation of toxin specificity to host specificity is complicated by the wide host range of the fungus and the different diseases that are caused. Toxin specificity may be presumed to relate only to the specific ability to cause a certain type of disease, that is seedling chlorosis. Certain plants, such as corn, show sensitivity of isolated chloroplasts but not of intact leaves, so that other factors may operate to cause insensitivity. In addition, the symptoms of chlorosis are most strikingly seen in seedlings with developing chloroplasts. The details of the causal relationship between inhibition of photophosphorylation and

this chlorosis are not yet established. Nevertheless, tentoxin could clearly become the first disease toxin for which the molecular basis of both specificity and toxicity can be explained. □

Coming to terms with the noisy retina

from Jonathan Ashmore

An international symposium on Photoreception was held at the Royal Society on September 2 and 3, 1976. Sponsored by the Rank Prize Funds, the symposium was organised by P. Fatt and H. B. Barlow.

RETINAL physiology has come to possess a decided flavour of engineering. Touching on problems close to the heart of any communications engineer, the mechanisms used by cells of the retina to transduce light signals in the presence of intrinsic noise were a recurrent theme at this meeting.

There now exist a number of complementary studies on the first stages of visual excitation, from the absorption of a photon by the photoreceptor pigment molecules to its transduction manifested as a change in membrane potential of the cell. There is still no evidence directly in conflict with the 'Calcium Hypothesis' advanced some years ago by W. Hagins and coworkers, (*A. Rev. Biophys. Bioeng.*, **1**, 131; 1972), which postulates that in rods calcium is released by light from the intradiskal space to diffuse intracellularly and close ionic channels on the cell membrane. Equally, there is no direct evidence supporting the idea, but Hagins and S. Yoshikami (National Institutes of Health, Bethesda), however, think that calcium is still the best bet for the intracellular transmitter. At this meeting they reported experiments intended to change the intracellular ionic environment in rat rods by fusing vesicles containing chelating agents with the cell wall. Model calculations suggest that calcium is the only ion with a sufficiently high affinity in these experiments to account for the observed changes in the rods' electrical photoresponse. Direct intracellular injection experiments in the toad rod system were reported by L. H. Pinto (Purdue University), J. E. Brown and J. A. Coles (Vanderbilt University), but again produced no evidence contradicting Hagins' story.

More than half the symposium was given over to the interactions between receptors and other cells of the retina. Since the initial indirect evidence 18 months ago by G. L. Fain that toad rod receptors are coupled (*Science*, **180**, 1178; 1975), the demonstration of inter-receptor coupling in various species has become almost a minor cottage industry. W. G. Owen and D. R. Copenhagen (University of California, San Francisco) discussed double impalement experiments with microelectrodes, showing conclusively that the coupling between rods in the snapping turtle is electrical, probably by way of the telodendria extending from the pedicles, (see *J. Physiol.*, **259**, 251; 1976). The effect of this coupling is to attenuate the signal from a singly illuminated rod to 10–20 times that produced when all the receptors are illuminated. One function for the coupling, at least in the peripheral retina, would be to improve the signal-to-noise ratio for dim diffuse lights, which would be traded off against the loss in visual acuity caused by coarsening the receptor mosaic.

Intracellularly recorded noise in turtle cones, probably produced by the opening and closing of ionic channels, has been used by T. D. Lamb and E. J. Simon (University of Cambridge) to show that in this animal the cones are electrically coupled. Quantitative measurements of the noise lead them to conclude that in these cells one photon can close one ionic channel, whereas in the rod system, as E. A. Schwartz (Harvard Medical School) reported using the same power spectrum methods, a figure closer to one photon controlling 300 channels may be more in order.

A nice bridge between electrophysiology and behavioural studies was made by G. L. Fain (Jules Stein Institute, UCLA). Using data from the behaviourally determined increment threshold curve for turtle and comparing this with the intracellularly determined light sensitivity, he estimates that a 5–10 μV signal is required in red cones for a behavioural response. This figure is below the cone noise level, although many parallel pathways exist in the retina for output. The properties of some of these retinal pathways have been mapped by D. A. Baylor and R. Fettiplace (Stanford University) by injecting current from a micropipette into cones and recording the ganglion cell response. They suggest that the pathways studied in the cone system may afford some signal-to-noise improvement by functioning as a band-pass filter; in the rod system at threshold where the problem becomes more acute a similar characteristic could be present.

The ionic mechanisms involved in

synaptic transmission have proved difficult to disentangle since usually more than one cell type meets the receptor at synaptic contact. At the synapse between receptor and depolarising bipolar cells, J-I. Toyoda (Kawasaki University) describing experiments to pass current trans-retinally produced evidence that at least two postsynaptic ionic channels are transmitter modulated. The hyperpolarising bipolar cells still conform to the original suggestion that light produces a permeability decrease to a single ion species.

A method to decouple the horizontal cells from their putative role in the feedback loop at the receptor triad synapse would be very helpful here, but failing this an interesting technique was reported by Yu. A. Trifonov and A. L. Bykov (Moscow University) to study the electrical properties of the horizontal cell membrane. Because of extensive electrical coupling between these cells, microelectrode studies of the cell membrane are not feasible; the method used here is a modification of Kramer's 'Winkel rinne', and results suggest cell membrane possesses anomalous rectification properties. Whether this will yield further information on the receptor synapse remains to be seen.

We may now be in a position to localise the cellular origins of the 'noise' treated in the psychophysical literature as the ultimate determinant of visual discrimination. Stressing this point, however, H. B. Barlow (University of Cambridge) tentatively suggested from experiments using random dot patterns that the factors which limit the quantum efficiency of seeing determined from performance tests may not arise in the retina at all but from "nasty central processes going on". Central limitations to quantum efficiency can only revise estimates of retinal efficiency upwards. □

Pesticides and the environment

by Mary Lindley

A symposium on the ecological effects of pesticides, sponsored by the Institute of Biology and the Linnean Society was held in London on September 23-24. The proceedings will be published by Academic Press.

clearly can be misused and have caused death and destruction in some cases. DDT and its successors have wrought so much good in controlling disease and increasing crop productivity that they must continue to be used, albeit in moderation and with constant efforts to find alternatives. This was the message of the symposium, during which very few speakers discussed directly the ecological effects of pesticides.

According to H. C. Gough (Ministry of Agriculture, Fisheries and Food, London), the benefits of pesticides used correctly in agriculture far outweigh their hazards. Their use has greatly reduced losses of many crops from pests, diseases and weeds. Without pesticides, he said, production would decline and costs would increase. Unnecessary use occurs, however, and there is a continuing need to ensure that hazards are minimised in the application of pesticides.

A particularly hopeful development in crop protection from the ecological point of view is selectivity. I. J. Graham-Bryce (Rothamsted Experimental Station, Harpenden) cited in particular the pyrethroids, which are potent insecticides, through their actions as nerve poisons, but not harmful to mammals. Another target for selective pesticides is the endocrine system of insects; juvenile hormone analogues and anti-juvenile hormone compounds hold promise.

The control of pests and diseases in forests presents a greater dilemma than when agricultural crops are concerned. Pesticides are in any case more likely to upset biological equilibria in perennial than in annual plant environments and the multipurpose nature of many forests adds to the complications, as M. J. Way (Imperial College Field Station, Silwood Park, Ascot) explained. He pointed out that the balance between species in forests may indeed be maintained by pests: for example, without spruce budworm some forests would be monocultures of spruce. But on the whole, pesticides are used less in forestry than in other major production systems.

The disastrous ecological consequences of the use of herbicides to defoliate large tracts of forest in Vietnam during the recent war were described by Dr A. H. Westing (Stockholm International Peace Research Institute). Severe destruction of habitats was caused in more than 50,000 hectares of upland forest and in 150,000 hectares of mangrove forest during the American programme of spraying with 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. According to Westing, the damage will be long lasting and has effectively deprived Vietnam of a proportion

of its forests.

The difficulties of monitoring the ecological effects of pesticides, particularly in developing countries, were made very clear by W. E. Kershaw and M. Pugh Thomas (University of Salford). They have been studying the effects of insecticides used to control the blackfly vector *Simulium damnosum* in the World Health Organization Onchocerciasis (river blindness) Control Programme. They have found that investigations of the biological side effects of pesticides on fast flowing rivers in Africa are expensive, tedious and hazardous. So far they have no evidence that fish have been affected, while the data obtained for invertebrates are very hard to interpret. Pugh Thomas believes that to allow for seasonal effects it will be necessary to compare data for successive years before a clear picture is likely to emerge.

The problems involved in predicting how pesticides will affect the environment were underlined by F. Moriarty (Institute of Terrestrial Ecology, Monks Wood Experimental Station, Huntingdon). Direct field trials are difficult, and the various indirect approaches used have disadvantages. But he said two tentative assumptions are possible: organisms have little effect on the amounts of pesticide in the physical environment, and if individual organisms are affected adversely, then ecological effects are imminent or already happening.

The prospects for wildlife, according to N. W. Moore (Nature Conservancy Council, London), depend more on the future of their habitats than on the use of pesticides. The decline in the wildlife population in Britain during the past 150 years has been due more to the destruction of habitats for land development than to the effects of pesticides. He pointed out, however, that treatment with pesticides can be locally damaging, and it will be increasingly important to ensure that pesticides do not spread away from the crops which they are protecting, for example by drift or in effluent. He said that the future of wildlife in the farmed countryside will depend on the conservation of areas of unfarmed habitat, where pesticides cannot reach.

Discussing future prospects for man, K. Mellanby (Institute of Terrestrial Ecology, Monks Wood Experimental Station) said that although in an ideal world pesticides would be unnecessary, they will continue to be used, and with proper precautions and controls, they can do their job while man's environment continues to be improved. He said that the dangers inherent in the use of pesticides are generally accepted and there has often been public overreaction to them. □

PESTICIDES are not likely to be abandoned to the accompaniment of cries of ecological doom, although they

articles

Astronomically-oriented markings on Stonehenge

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In view of the years of careful study that have been devoted to Stonehenge it may seem unlikely that any more information could be extracted from the monument, or at least from that part of it above ground. During the past two years, however, further markings have come to light that are, at least, interesting, and possibly important. These markings are a series of at least 11 pits on the upper surfaces of the three contiguous lintels (130, 101 and 102) that span the well-known line of sight from the centre of the sarsen circle north-eastward towards the heel stone. For an observer diametrically across the circle, 9 of these pits identify directions of the rising moon at significant points in its 18.6-yr cycle.

THE lintel-top surfaces of Stonehenge maintain a true level with the horizon to within a few inches in spite of the fact that within the sarsen circle the ground level drops down towards the north-east by ~ 1.5 feet. If the sole purpose of the lintels was architectural, it is hard to see why such a small correction-to-level would have justified the effort; its architectural effect would be imperceptible. But as a walkway for observing horizon events such a correction would make sense. Moreover, the diminutive, upright stone 11 in the south-east quadrant could then be explained as a mid-way support to a stair which, forking to left and right at its top, gave access to the lintel-top walkway.

This hypothesis suggests the possible existence of astronomically oriented markings on the lintel surfaces, but nowhere in the Photographic Library of the Department of the Environment was there a close-up picture of any of the nine lintels now in place. Direct inspection was therefore necessary.

In July of 1974 with the permission of the Department of the Environment and the help of Mr Woodhouse, the head custodian, a ladder was provided and all nine lintels

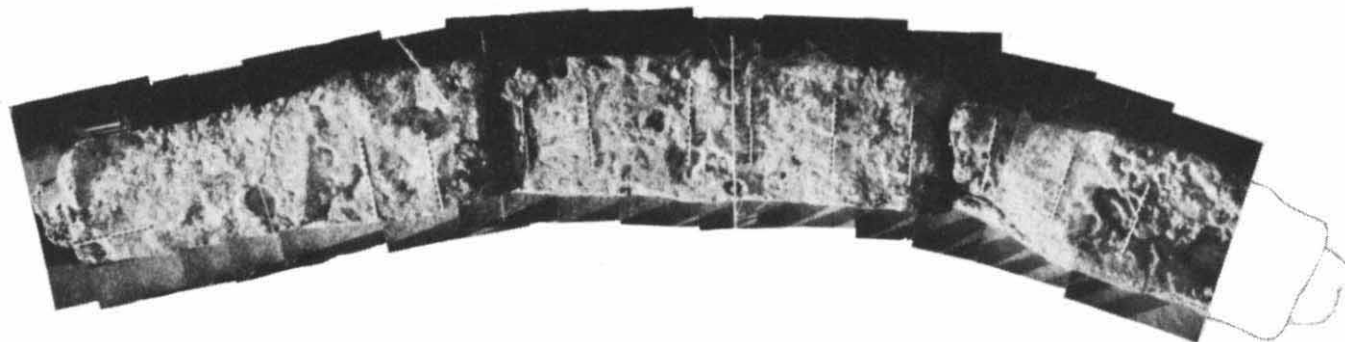
inspected. Only lintels 130, 101, and 102 (the three contiguous ones in the north-east quadrant of the sarsen circle) showed promising markings'. In 1975, again with permission and help, a series of measurements, photographs, and plasticene moulds were carefully made. Figure 1 shows the surface of the three lintels made by piecing together a sequence of photographs made approximately every 2 feet along the lintel tops from a height of 5.5 feet. They were taken with the Sun low in the west, at about 1800 on June 13, 1975, shadowing the rock surface. The measuring stick is 2 feet long.

Pits

The significant markings are a series of pits, some of which in the photograph contain vertical wands used for locating them precisely from the ground. The pits are identified by numbers in Fig. 2, which is made from a tracing of a photograph, originally 2.5 feet long.

Pit 1, the largest, is almost circular both in plan and in section, ~ 10 inch across and 3 inch deep. (The numbers here given are only approximate because of the irregular surface of the lintels, and the fact that not all the pits have clearly defined edges.) Pits 2, 3 and 4 are much smaller, 1.5–2 inch in diameter, quite round but irregular in depth. Pit 5 is round, 3 inch in diameter and about as deep. Near it is a pit, 6, that may be more significant though it is of irregular shape, an uneven 3×1.5 inch in plan and up to ~ 2 inch deep. Pits 7 and 8 command attention because of their location almost directly over the sight line from the sarsen circle centre to the heel stone. They are both approximately 4 inch in diameter and depth with regularly rounded bottoms. Finally, pits 9, 10 and 11 are shallow, smaller, and not quite so regular in shape. Pits 1 to 8 (excluding 6) are so regular in shape as to be plausibly man-made; the others together with several smaller markings command less confidence, but all of them stand out sharply in appearance from the uneven weather-worn surface of the lintels. Indeed, pits 1, 7 and 8 can be detected in some low level air views already in print.

Fig. 1 Composite photograph of lintel tops from a height of ~ 5.5 feet showing pits. The measuring stick is 2 feet long.



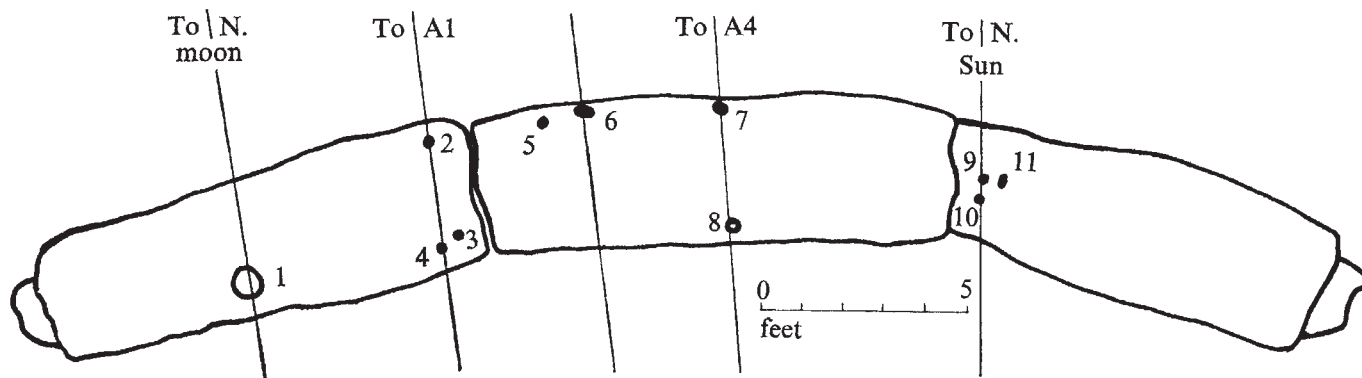


Fig. 2 Identification of pits in Fig. 1 and the alignments through them. The directions shown are approximate.

Alignments

To pursue the possibility that the pits are man-made it is necessary to locate them accurately on the overall plan of the monument. It was found by projecting Hawkins's 1965 photogrammetric air survey map² on to an enlarged drawing of the Thoms' more recent survey³ that the two agree precisely in all essentials. We used the air survey, except for the replacement of the three lintels by a reduced rendering of Fig. 2.

Nine of the eleven pits identify significant directions for an observer posted on the now non-existent lintel top diametrically across the sarsen circle at P (Fig. 3). P falls on the centre of sarsen upright 15.

When the lintel-top pits are plotted on the air survey map it is found that a line through pit 1 to the northernmost point of moonrise and a line through pits 9 and 10 to the northernmost (midsummer) sunrise intersect on the south-western side of the sarsen circle at P. P is defined by the intersection of these two sight lines.

Moreover, as viewed from P, pits 2 and 4 point very nearly but not precisely to the northernmost of the four A holes (A1). Similarly, a line from P through pit 5 (not drawn in Fig. 3) points very nearly to A2. A line from P through the Aubrey hole centre shown as a dot (but not through the sarsen circle centre shown as X) passes through pit 6 and very close to the midpoint of the A holes between A2 and A3. And the conspicuous pits 7 and 8 over the central archway point very nearly to A4. All of these lines miss the A holes by the same amount and in the same direction (eastward), a matter discussed further below. Only pits 3 and 11 elude an obvious pattern. So simple a pattern

fitted so precisely by such a large majority of the pits is additional evidence that the pits (or at least nine of them) are man-made.

From point P none of the pits aligns with the rising point of Venus or of any of the 20 brightest stars except, implausibly, Pollux after corrections are made to the year 2000 BC for the precession of the pole. Nor do any of the pits seen from the sarsen circle centre or the Aubrey hole centre align with any significant sunrise or moonrise points or with Venus or the brightest stars.

A shift in the observation point

There is a strong intuitive appeal in the idea that the viewing point should not be at P but at a point ~5 feet westward so that the observer would be on what the Thoms³ have identified as the axis of symmetry of the monument. There he would have a clear view under the lintel of the great trilithon whose bottom surface would be 5.5–6 feet above his footing atop the sarsen circle. The angular spread of the alignments (pit 1 to pits 9, 10) is 10°, too great to be seen through the trilithon from one spot, but as the observer walked north-westward from P along the lintel tops he would see the pits revealed progressively through the arch as he moved. Pit 1 would come into view (azimuth 42.3°) in approximately the direction of the northernmost moonrise (azimuth 40.5°), and pits 7 and 8 would be seen almost (but not quite) in line over the sarsen circle centre, with the centre of the heel stone at 51.7°; the azimuth of the midsummer sunrise is 50.7°. These are unsupportably large discrepancies. Moreover, viewed through the trilithons in this way pits 2–6 and 9–11 have no obvious significance.

Fig. 3 The alignments through the lintel-top pits to outlying A holes and horizon points.

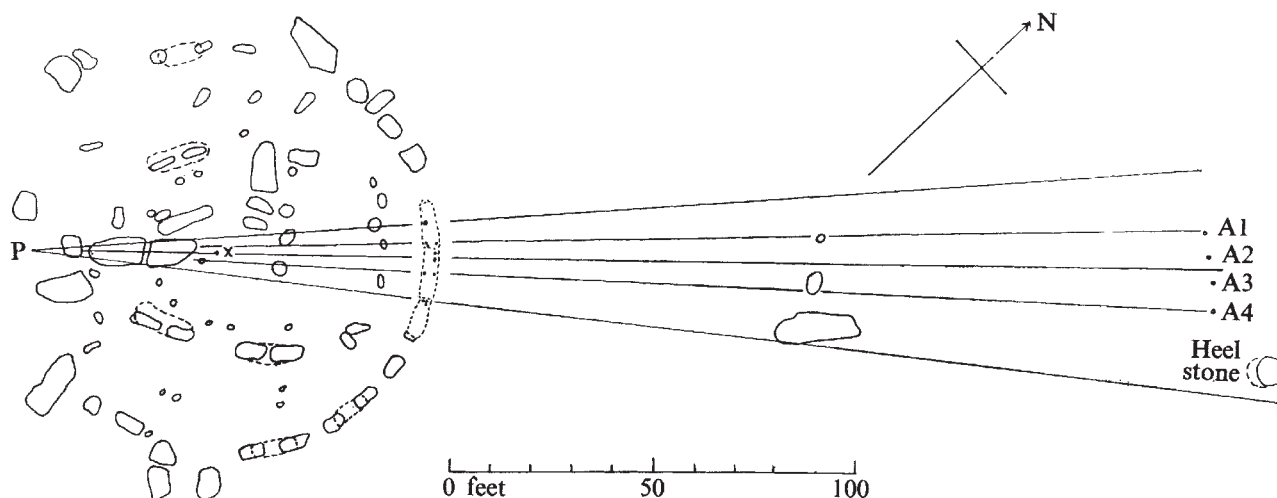




Fig. 4 Close-up of some of the pits showing how they may have been used to support wands as sights.

Also, when vertical wands are erected in pits 2, 4 and 7, 8 (Fig. 4) they appear precisely aligned only as seen from within ~ 1 foot from P (Fig. 5) and not through the trilithon arch, 5 feet from P. Finally, since the axis of the monument is aimed very nearly at midsummer sunrise, it can scarcely be aimed simultaneously at, or be symmetrical with, moonrise points all to the N. On the whole, the alignments radiating from P are impressively accurate.

Orientation

With the help of plumb lines pit 1 was located at ground level on the monument plan to within 4", and its azimuth was found to be 40.5° . Using the procedure given in ref. 4 the first gleam of the northernmost moonrise ($\delta = +29.1^\circ$) on the horizon of Stonehenge is found to have an azimuth of 40.7° .

Similarly, the ground level location of pits 9 and 10 identify an azimuth of 51.0° from P, whereas the first gleam of midsummer sunrise ($\delta = +23.9^\circ$) seen over the heel stone from the sarsen circle centre has an azimuth of 50.1° .

With pits 1, 9 and 10 thus located on the plan, a tracing of the lintel-top photograph (Fig. 2) was reduced in size until pits 1, 9 and 10 on the photograph registered accurately with the plan. The lintel-top tracing was then drawn, thus locating accurately all the intervening pits. So located, the pits identify the lines toward the A holes (Fig. 3) (See Table 1 for azimuths).

The accuracy with which the sunrise and moonrise lines are located on the map is limited by uncertainties of an inch or two in the restoration of the three lintels in 1919–20 and the fact that location of the pits on the map are accurate to only $\sim \pm 4$ inch and that angles measured in Fig. 3 are no better than $\pm 0.25^\circ$. Together these errors result in an uncertainty in the location of P of ~ 1.0 foot.

Accuracy

In an effort to judge the accuracy with which the lintel-top pits align with the A holes, a camera was set up approximately at point P. In Fig. 5 beneath the right-hand lintel top wands in pits 7 and 8 is an arrow pointing to a post erected in the centre of hole A4, almost exactly three sarsen circle diameters from the camera. Although pit A1 is obscured it can be located in effect by simple geometry 20.4 feet left of A4, which agrees with the distance actually measured on the ground. Measurements on the photograph show that to make the pits align with the A

holes, the camera would have to move 1.0 foot eastward to be centred on P and then an additional 1.4 feet eastward beyond P. This is appreciably more than the ± 1.0 -foot uncertainty, inherent in the original map location of P. Thus, in spite of their identity of spacing, the set of sarsen circle alignments appears to be shifted slightly to eastward of the set of A-hole alignments. It looks as if the builders had come up with two slightly different solutions to the same observational problem. For an explanation of this mismatch we must first examine the consequences of the fact that the A holes and the sarsen circle lintel pits were in use at two widely different times.

Stonehenge I

The A holes 3 feet in diameter, were only 4 feet deep, much too shallow to support the 28-foot posts required for a man standing on the lintels to use them for sighting. Moreover, post hole A1 was not only filled in but was entirely buried by the bank created by the building of Stonehenge II. Together these facts point to the use of the A-hole posts as sights by ground-level observers before the construction of Stonehenge II.

At that early time, when there was no sarsen circle, it seems likely that the observers were stationed at the centre of the Aubrey hole circle rather than at P. The reason for this is simply that from the centre of the Aubrey hole circle the four A-hole posts (centre line azimuth 45.5°) would have been centred between the line to the heel stone (50.7°) marking the northernmost sunrise, and the line to the northernmost moonrise (40.5°). Posts on the A holes would then mark the 18.6-yr swing of the moon during intermediate years. When the Moon rose through the first opening (A4–A3) of the four posts, it was just one quarter of the way in time from rising on the line of midsummer sunrise and rising at its northerly extreme; when it rose through the middle opening it was just one third of the way to its maximum, and when it rose behind A1 it was exactly halfway.

Although the precise date of the maximum would be impossible to identify accurately, just as for the Sun, since, close to the maximum, the change in azimuth with time is too small to observe, the date could nonetheless be inferred quite accurately in retrospect as lying halfway in time between the northbound and the southbound traverses of any one of the three A-hole openings. After one or two such pairs of traverses (18.6 yr apart) the observers could confidently predict the proper date for ritual celebrations of the maximum.

Fig. 5 Ground-level view of the wands in Fig. 4 from a point 1 foot west of the presumed position of P.

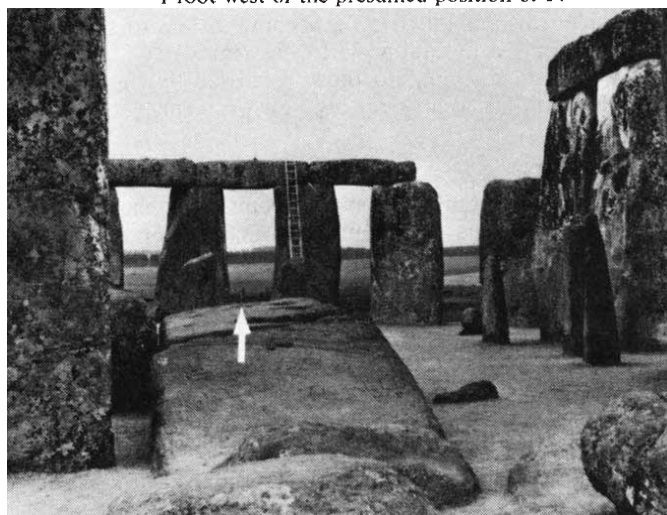


Table 1 Orientation data of the pits on Stonehenge lintels

Alignment from P through pit:	Towards	Azimuth E of N $\pm 0.2^\circ$	First gleam	Vertical error, D°, for Centred on horizon	Tangent on skyline
1	Northernmost moon $\delta = +29.1^\circ, A = 40.5^\circ$	40.5	-0.09	-0.39	-1.67
2, 4	A1	43.5			
3		43.6			
5	A2	44.5			
6	Mid-A and Aubrey centre	45.5			
7, 8	A4	47.5			
9, 10	Midsummer sunrise $\delta = +23.9^\circ, A = 50.7^\circ$	50.7	+0.63	+0.40	+0.10
11	Midsummer sunrise	51.2	+0.92	+0.68	+0.45
Through centre of great trilithon to pit:		Azimuth E of N $\pm 0.6^\circ$ *			
1	Northernmost moon $\delta = +29.1^\circ, A = 40.5^\circ$	42.3	+0.75	+0.52	+0.29
2, 4		45.8			
3		47.5			
5		47.8			
6	Midsummer sunrise	48.5	-0.53		
7	Midsummer sunrise	51.7	+1.18	+0.94	+0.82
8	Midsummer sunrise $\delta = +23.9^\circ, A = 50.7^\circ$	51.8	+1.23	+0.99	+0.87
9, 10		56.0			
11		56.5			

*Error is higher than from P because of uncertainty concerning midpoint of trilithon arch.

The end came when the Beaker invaders, bringing with them a new culture, realigned the axis by the construction of Stonehenge II, evidently focusing on the midsummer sunrise and burying the A holes. The Moon seems to have been replaced by the Sun in the culture of the newcomers.

Stonehenge III

Centuries after the A holes had been obliterated the sarsen circle was erected, its axis also oriented to the midsummer sunrise, as was Stonehenge II before it. Then or later, wands supported upright in the new lintel-top pits must have once more identified the old alignments for observers at P, now 16 feet up off the ground to avoid being blocked by the new sarsen circle and the old bluestone circle uprights.

In their paper⁵ on Stonehenge as a possible lunar observatory the Thoms discuss the improvement in visibility of foresights on distant hills made possible by raising the level of the observer above the ground level. Is this consideration one of the determinants of the height of the lintel-top walkway? No one has ever explained convincingly why it had to be 16 feet high. Do our pits identify distant foresights visible only to a lintel-top observer? The Thoms mention C. A. Newham's⁶ observation that if Stonehenge were raised only a few feet, distant hills would become visible to the north-east. Certainly a lintel-top platform creates new possibilities for observations similar to those discussed by the Thoms^{3,4} and Newham⁶ as well as raising new problems.

Difficulties

One problem that has a plausible solution is the mismatch between the A-hole alignments and the lintel-top pits alluded to earlier and illustrated (and exaggerated) in Fig. 5. Their alignments shifted slightly eastward, the lintel-top pits appear to attest to the slow eastward drift of the extreme declinations of the Sun and Moon caused by the slowly changing obliquity of the ecliptic. For example, in 3000 BC the maximum declination of the moon was 29.18° , and in 2000 BC it was 29.08° (ref. 7). As a consequence the northernmost rising point of the Moon shifted 0.21° eastward. But a 0.21° shift eastward corresponds to the observer moving 0.5 feet eastward from the presently plotted position of P. This, together with the 1.0-foot uncertainty in P's

location amounts to 1.5 feet, a shift in P sufficient to bring the two sets of alignments into precise coincidence, assuming the maximum possible error in the original location of P. (A 1.4-foot shift in the map position was required.) In principle, if the position of P were known exactly and our interpretation is correct, the angular difference between the two sets of alignments would lead to a knowledge of the time difference between Stonehenge I and the creation of the lintel-top pits on Stonehenge III.

A much more serious problem remains. When Stonehenge III had been completed stone 55 of the great central trilithon, now fallen, would have completely blocked the view of the lintel-top observer at P. Perhaps the trilithon was erected only after the sarsen circle and the pits had been in use for some time, but this seems unlikely since it would be almost impossible to erect the trilithon after the sarsen circle had been completed. Nor do our pits seem to identify any useful alignments to an observer looking through the trilithon arch.

Speculations

Perhaps in the troubled times towards the end of the use of Stonehenge as an observatory, the trilithon was pushed over—to make way for the re-establishment of the lunar alignments—and has lain broken in its present position ever since. If this event is archaeologically dubious, it at least has the merit of accounting for most of the pits.

More plausibly, perhaps, a 4-foot high wooden platform may have been erected at P to permit viewing over the top of the trilithon.

In conclusion, although we lack an exact explanation of how sightings could have been made or why the alignments were off-axis, these results nevertheless have significance in our understanding of the astronomical aspects of Stonehenge. The original theory that the site was an observatory accurate to the width of the archways is now augmented with alignments accurate to within a few inches.

For encouragement, advice, and active support in this work I wish to thank Gerald S. Hawkins, G. W. Cottrell, John M. Saul (who took Figs 4 and 5) and Thomas Woodhouse, the head custodian of Stonehenge, but above all the kindly fate that so improbably spared the three contiguous lintels bearing the evidence.

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²⁰⁷Pb/²⁰⁶Pb whole-rock age of gneisses from the Kangerdlugssuaq area, eastern Greenland

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We present here a brief description of geological relations and results of lead isotopic analyses of eight whole-rock samples of Precambrian gneiss from an area near the Skaergard Intrusion in the Kangerdlugssuaq region of eastern Greenland.

EXCEPT for Wager's¹ regional reconnaissance studies, there have been few published details of the Precambrian geology of Eastern Greenland. Berthelson and Noe-Nygaard² have suggested that basement rocks of the Kangerdlugssuaq region belong to the Kenoran tectonic division (that is, rocks of pre-Ketilidian, or more than about 1,800 Myr old) of the Canadian-Greenlandic Shield. A limited number of K-Ar ages³⁻⁴, ranging from 2,700 to 1,600 Myr BP, have been reported for Kangerdlugssuaq area gneisses, but interpretations of these data are suspect because of widespread Tertiary igneous activity in the area⁵⁻⁷. Our field studies have indicated a complex history for the gneisses, which is summarised in Table 1.

Interlayered mafic and felsic gneisses represent the earliest recognisable unit in the area (event 1, Table 1). These interlayered gneisses are interpreted as dominantly metaigneous rocks, but a small amount of intercalated metasedimentary material is locally present. Strong banding is developed in the gneisses primarily among quartzofeldspathic units. Distinctive mafic bands a few centimetres to several metres thick are also present. This gneiss suite has characteristics similar to those described for gneisses in western Greenland and equivalent gneisses elsewhere in the Archean Craton of the north Atlantic region⁸⁻¹⁰. In the Kangerdlugssuaq area mafic gneisses vary from nearly pure hornblende to plagioclase-rich amphibolite. Locally, this lithology is abundant, forming mappable units of mafic gneiss up to 200 m thick. More commonly, the mafic gneisses have been pulled apart, rotated, and fragmented within a matrix of quartzofeldspathic gneiss, giving the impression that large mafic gneiss xenoliths have been rafted and deformed by later emplaced felsic gneisses. This impression may, however, be an oversimplification in as much as the mafic gneiss blocks are in places definitely interlayered with quartzofeldspathic to biotitic gneisses. Thus, it seems probable that some blocks of felsic gneiss also occur as xenolithic rafts.

Metasedimentary rocks which occur among the early gneisses are dominantly felsic gneisses with variable amounts of plagioclase, alkali feldspar, and quartz and accessory biotite and hornblende. With increasing quartz content these gneisses grade to sugary textured quartzites; with increasing biotite they become schistose and some contain garnet porphyroblasts in a fine-grained quartz and feldspar matrix. Hornblende-rich and/or biotite-rich layers are also present in the felsic metasedimentary gneisses. It

is possible that these metasediments are remnants of a once extensive supracrustal sequence that was incorporated into the metaigneous suite either as conformable sedimentary beds in a volcanic sequence or as infolded units. Alternatively, they may reflect conformable tectonic interleaving, as inferred for the Malene supracrustals within the Amitsoq gneisses in Godthabsfjord⁸.

Textural and structural relationships within the later sequence of metaigneous rocks (event 2, Table 1) suggest that there were several intrusive pulses. The early intrusive rocks are commonly granite or trondhjemitic. Because of their similarity in composition and texture to felsic rocks of the earlier interlayered gneisses, contacts between the two units are discernable only in a few favourable localities. Discordant contacts are most clearly displayed where rafted blocks of agmatized interlayered mafic-felsic gneiss are enclosed by intrusive granitic or trondhjemitic gneisses. Locally, the metaigneous gneisses display pronounced coarsening, largely conformable with foliation, and associated with the occurrence of pegmatitic patches and lenses; in extreme cases these zones become essentially migmatitic. It is not clear whether the coarsening effect and migmatitisation are related to a separate intrusive pulse. In some areas granitic rocks occur with little internal fabric; these bodies are apparently the latest recognisable intrusions in the area.

The grade of metamorphism throughout the entire gneiss sequence is amphibolite facies, except in areas where migmatitisation is most widespread and/or intrusive rocks are more abundant. There, pyroxenes are present rather than biotite and hornblende in the felsic gneisses, and the grade of metamorphism reaches granulite facies. The higher metamorphic grade may be a relict of the cooling history of the intrusive bodies.

Table 1 Brief outline of geological events recorded in the Kangerdlugssuaq region, eastern Greenland

- 1 Accumulation of interbedded volcanic and minor sedimentary units. Metamorphism and deformation produced interlayered mafic and felsic gneisses, strongly folded isoclinally.
- 2 *a*, Intrusion of granitic and trondhjemitic plutons accompanied, or closely followed by, strong deformation and recrystallisation which produced disharmonic folding of the interlayered mafic and felsic gneisses.
b, Igneous activity was apparently protracted and in later stages produced mafic as well as potash-rich granitic plutons and pegmatites.
- 3 Subsequent to, and perhaps closely following, intrusive activity all the layered gneisses and plutonic bodies were folded and metamorphosed. This was followed, possibly at the end of folding, by pervasive shearing and by partial retrogressive recrystallisation.

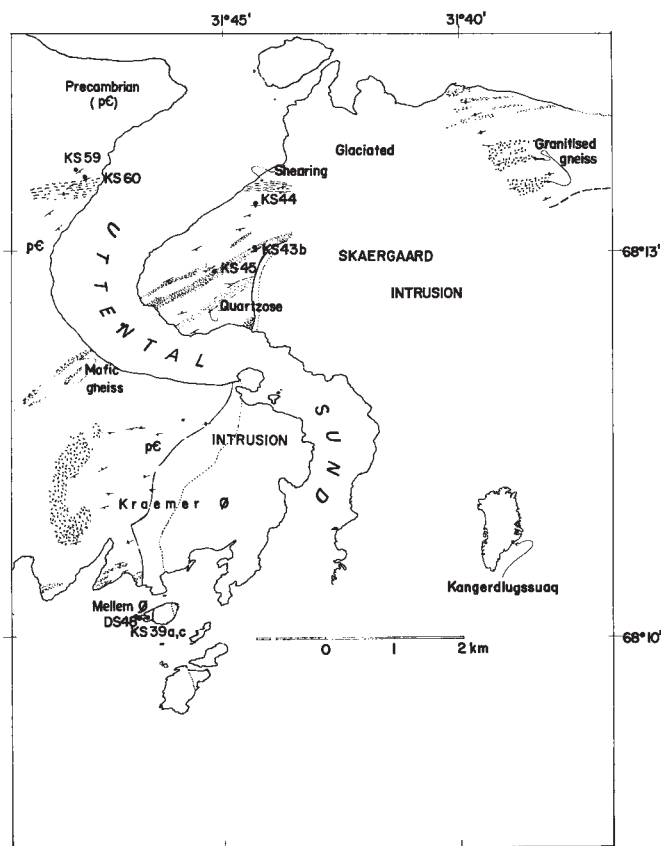


Fig. 1 Geological sketch map of the Kangerdlugssuaq area, showing locations of analysed samples.

Deformation of the gneisses is intense, with at least three major episodes of folding accompanying the several intrusive pulses. In the Kangerdlugssuaq area pervasive shearing (event 3, Table 1) has affected the continuity of earlier fold forms. Shearing was also accompanied by partial retrogressive recrystallisation. In areas of very intense shearing recrystallisation was more complete, pegmatitic gneisses were deformed to augen gneisses, and finer-grained rocks were mylonitised.

Lead isotope results

Samples from each of the gneiss sequences were selected for lead isotopic analysis; sample localities are shown in Fig. 1. Whole-rock powders were dissolved with HF and HClO₄, and lead was extracted using an HBr cation exchange procedure. Full procedural blanks amounted to 10–15 ng for 1-g samples (that is, less than 1% of the total quantity of Pb processed for any sample). Isotopic analyses were made on a 12-inch solid source mass spectrometer, using a silica gel–H₃PO₄ matrix. All analyses have been corrected for mass fractionation in the spectrometer by intercomparison with the NBS common lead standard (SRM-981). Corrected isotopic ratios are believed to be accurate to better than 0.15% of absolute values.

The isotopic analyses (Table 2) for the Kangerdlugssuaq gneisses are shown in a ²⁰⁷Pb/²⁰⁴Pb–²⁰⁶Pb/²⁰⁴Pb diagram in Fig. 2. If the inconsistent sample KS-60 (cataclasised augen gneiss) is excluded, the remaining seven samples lie within analytical uncertainty of an isochron with a slope of 0.220 ± 0.003 (1σ), corresponding to an age of $2,980 \pm 20$ (1σ) Myr BP (see ref. 11). Such a close fit to the Pb–Pb isochron suggests that these gneisses were derived from a source region that was homogeneous with respect to U/Pb during approximately single-stage evolution from the time of formation of the Earth to the isochron date. Geochemical differentiation at the latter time produced severe fractionation of U/Pb as well as isotopic homogenisation of Pb (that is, igneous parental material for

the gneisses acquired the mean isotopic composition of their source region at the isochron date, but had variable U/Pb). It is not clear whether isotopic rehomogenisation took place during the final metamorphism of gneisses emplaced over a significant period of time, or whether the gneisses were derived from the same homogeneous source over a relatively short period of time just before the culminating metamorphism. In the first case, the isochron date represents event 3 in Table 1, and a significant history of unknown duration is implied for the earlier events. In the second case, the isochron date may reflect the age of igneous material that was parental to the gneiss complex. But because the rocks associated with each sequence of events conform to the isochron, the latter possibility requires that the culminating metamorphism closely followed (within a few 10⁷ yr) emplacement of the various parental rocks. We cannot yet resolve this problem, but because of the complex geological history recorded by the Kangerdlugssuaq rocks the first possibility seems more likely. Considering the wide spread in ²⁰⁶Pb/²⁰⁴Pb obtained for our samples, and the small residuals obtained in the isochron regression, it seems improbable that further whole-rock Pb isotope analyses of Kangerdlugssuaq gneisses will reveal ages older than about 3.0×10^9 yr.

Sample KS-60 may be inconsistent with the lead-isotope systematics of the other samples because of uranium loss (relative to lead) subsequent to intense shearing of this rock. But recent loss of uranium (for example during early Tertiary igneous activity or post-glacial uplift in the area) would not account for the comparatively low ²⁰⁷Pb/²⁰⁴Pb ratio in KS-60. It also seems unlikely that KS-60 was not initially affected by the event which caused effective homogenisation of lead isotopic compositions in the other gneiss samples (as seems required from the excellent fit of these samples to the ²⁰⁷Pb/²⁰⁴Pb–²⁰⁶Pb/²⁰⁴Pb isochron). Therefore, we think it likely that uranium loss occurred during, or shortly following, the shearing of KS-60.

Kangerdlugssuaq gneisses

A single-stage ²³⁸U/²⁰⁴Pb (μ₀) of 8.1 is indicated for the source materials of the Kangerdlugssuaq gneisses. This value of μ₀ corresponds to a lead isotope growth curve which intersects our ²⁰⁷Pb/²⁰⁴Pb–²⁰⁶Pb/²⁰⁴Pb isochron at 2,980 Myr and 0 Myr, based on values for primordial lead isotope ratios and decay constants given in ref. 11. This comparatively low value of μ₀ is compatible with a mantle or lower crust source region for igneous precursors to the gneisses, because upper crustal rocks are generally enriched in U/Pb. For example, such enrichment in U/Pb is inferred for the Isua supracrustal rocks which yield a μ₀ value of 8.7 (ref. 12). On the other hand, profound U depletion noted in many ancient infracrustal gneisses can

Table 2 Lead content and isotopic composition of Kangerdlugssuaq gneisses

Field no.	p.p.m. Pb	²⁰⁶ Pb/ ²⁰⁴ Pb	²⁰⁷ Pb/ ²⁰⁴ Pb	²⁰⁸ Pb/ ²⁰⁴ Pb
KS-39A (1)*	9.19	16.601	15.299	37.568
Mafic gneiss with felsic layers				
KS-39C (2)*	13.31	17.164	15.369	35.908
Felsic gneiss				
KS-43B (3)*	4.30	14.861	14.886	34.587
Porphyritic felsic gneiss				
KS-44 (4)*	1.47	17.670	15.556	36.677
Amphibolite gneiss				
KS-45 (5)*	20.03	14.061	14.730	33.407
Pegmatitic plutonic gneiss				
KS-59 (6)*	30.00	20.434	16.169	33.876
Kf-rich augen gneiss				
KS-60 (7)*	12.67	20.467	15.841	60.468
Cataclasised augen gneiss				
DS-48 (8)*	39.83	23.387	16.749	36.760
Kf-rich granitic pegmatite				

*Numbers used in Fig. 2.

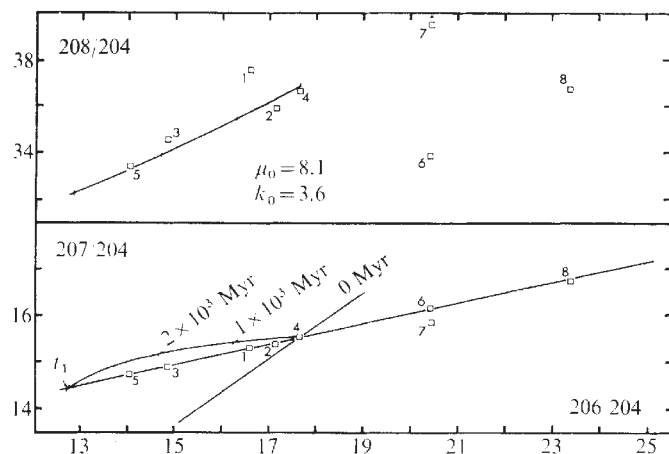


Fig. 2 Lead isotope systematics of analysed samples. A single-stage growth curve with a μ_0 value of about 8.1 is shown for reference. t_1 , 2,980 Myr.

result in present-day unradiogenic Pb isotopic compositions. For comparison, 2.8×10^9 yr-old granulite facies rocks from south-western Greenland yield a μ_0 value of 7.5 using the same decay constants¹³. The lower value of μ_0 obtained for these granulite gneisses is in accord with the observation that U depletion commonly becomes more pronounced as metamorphic grade increases¹⁴. The 3.7×10^9 yr-old Amitsoq gneisses of western Greenland also yield lower values of μ_0 (about 7.6 to 7.7) and, in addition, contain Pb that is much less radiogenic ($^{206}\text{Pb}/^{204}\text{Pb}$ ranges from 11.5 to 14.9)^{15,16} than the Kangerdlugssuaq gneisses. Consequently, it is unlikely that the Kangerdlugssuaq gneisses could represent metamorphosed parent material equivalent in composition to the Amitsoq gneisses. Our work does not preclude the existence in the Kangerdlugssuaq area of older material with a composition different from that of the Amitsoq gneisses. The Viken migmatitic gneisses in northern Norway yield a Pb-Pb whole-rock isochron age of about 3.5×10^9 yr, and a value of μ_0 of 8.9 (using the decay constants given in ref. 11)¹⁷. The range of $^{206}\text{Pb}/^{204}\text{Pb}$ values in these gneisses (14.8 to 21.2) is within the range for the Kangerdlugssuaq gneisses, but the latter display lower $^{207}\text{Pb}/^{204}\text{Pb}$ ratios in accordance with their younger age. Conceivably, high-grade metamorphism of Viken-like rocks more than 3.0×10^9 yr ago could have pro-

duced gneisses with Pb isotopic compositions like those in the Kangerdlugssuaq rocks. A discussion of the origin of the Kangerdlugssuaq gneisses will be presented elsewhere.

The approximately 3.0×10^9 -yr-old metamorphism recorded in the Kangerdlugssuaq gneisses was apparently related to regional metamorphism of high amphibolite or granulite grade (depending in part upon level of exposure) that affected much of the Greenland Shield between 3.0 and 2.7×10^9 yr ago^{9,13,18-23}. High-grade metamorphic rocks of similar age have been documented from the Lewisian Complex of north-western Scotland²⁴⁻²⁷. It has been noted that this blanket metamorphism affected supracrustal as well as infracrustal rocks, thus recording an actual rise in crustal temperature during that time⁹. The complex geological relationships recorded in the Kangerdlugssuaq gneisses, like those occurring in the Lewisian gneisses¹⁰, suggest a more extensive history of crustal evolution that predates the 3.0 – 2.7×10^9 -yr-old event. U-Pb zircon and Rb-Sr whole-rock analyses are in progress in an attempt to resolve earlier events and to test our interpretation that the whole-rock Pb-Pb isochron age for the Kangerdlugssuaq gneisses reflects the time of last major metamorphism in that area.

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Abnormal or absent β mRNA in β^0 Ferrara and gene deletion in $\delta\beta$ thalassaemia

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In patients with β^0 thalassaemia from Ferrara, β globin mRNA sequences are either absent or structurally abnormal while in β^0 thalassaemia in Catania, β globin mRNA sequences are present. In $\delta\beta$ thalassaemia there is a deletion of β -like globin genes, while in β^0 Catania DNA, no β globin gene deletion is detectable.

THREE general types of β thalassaemia have been described to date: β^+ thalassaemia, associated with the production of decreased amounts of structurally normal β globin; β^0 thalassaemia, characterised by the absence of β globin synthesis; and $\delta\beta$ thalassaemia, in which synthesis of both δ and β globin is absent¹. The defect in β globin synthesis in these syndromes can be demonstrated by measurements of globin synthesis both in whole cells and by addition of isolated mRNA to

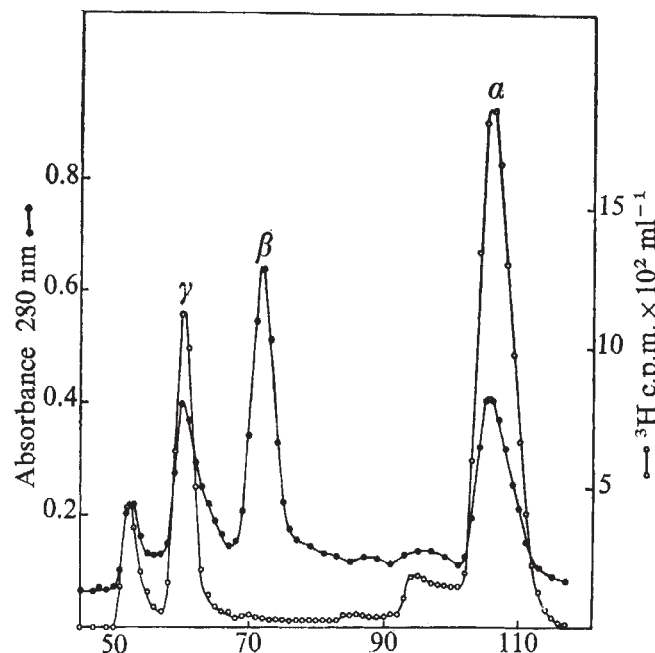


Fig. 1 Globin synthesis in reticulocytes of β^0 Ferrara. Elution from carboxymethyl cellulose chromatography of globin prepared from cells of a patient homozygous for β^0 Ferrara (patient 2 in Fig. 4b and Table 1) which had been incubated with ^3H -leucine.⁴ The patient had been transfused 22 d before the blood sample was obtained.

heterologous cell-free systems²⁻⁵. More recently, the availability of complementary DNAs (cDNAs) specific for α and β globin mRNA sequences has made it possible to quantify the α and β mRNA content of cells in these disorders⁶⁻¹². In the β^+ thalassaemias, we and others^{6,7} have shown previously, using these molecular probes, that the amounts of cytoplasmic β globin mRNA is decreased. By contrast, in β^0 thalassaemia in patients from Italy, there have been conflicting reports^{8,11} either of the absence of structurally normal β mRNA sequences, or of structurally abnormal β mRNA sequences. β mRNA sequences in $\delta\beta$ thalassaemia have been shown previously to be absent⁸. We report here that in β^0 thalassaemia in patients from the Ferrara region of Italy, another distinctive genetic defect may be reflected at the mRNA level by the inability of β^0 Ferrara mRNA to hybridise fully to β cDNA. In contrast, we find significant amounts of β mRNA in β^0 Catania mRNA, which is consistent with the presence of abnormal, untranslated mRNA in some β^0 thalassaemia patients^{6,11}. We also report that

in $\delta\beta$ thalassaemia there is a deletion of structural δ and/or β globin gene sequences; in β^+ and β^0 Catania DNA, no such deletion is detectable. These results indicate a heterogeneity of gene defects in β thalassaemia, all of which can lead to decreased or absent β globin synthesis.

The β^0 Catania patients are homozygous for β^0 thalassaemia and manifest the clinical symptoms of β thalassaemia. They range in age from 5 to 10. All of the patients have hepatosplenomegaly, abnormal red cell morphology with hypochromic, microcytic anaemia, elevated haemoglobin A₂ and F levels and require blood transfusions. The $\delta\beta$ thalassaemia homozygote is a patient who has only haemoglobin F, a mild anaemia, and thalassemic smear with hypochromic, microcytic cells¹³. The β^0 Ferrara patients have high A₂-type homozygous β thalassaemia¹⁴. Fibroblasts from patients with hereditary persistence of foetal haemoglobin (HPFH) and from normal patients were cultured by Dr Robert Krooth. The HPFH fibroblasts are from a patient who is homozygous for the black form of this disorder¹⁵.

Total RNA was extracted from peripheral blood samples from which buffy coat had been removed¹⁶. RNA was fractionated on sucrose density gradients and the 6-16S region was used in hybridisation and biological activity studies. α and β globin cDNAs were purified as previously described⁹. The α and β globin cDNAs sediment as a relatively homogeneous 7S peak using alkaline sucrose gradient analysis. DNA was prepared essentially as described previously¹⁷. Hybridisation of globin cDNA with spleen, liver, fibroblast and white blood cell DNA was also performed as previously described⁹.

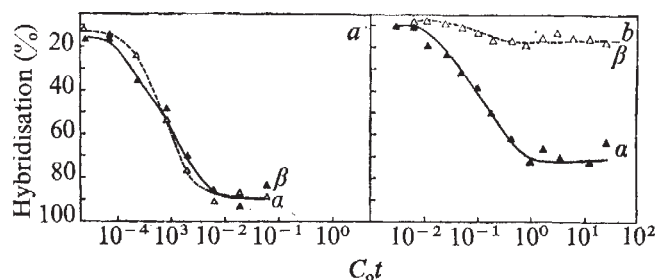


Fig. 2 Hybridisation of α and β cDNA with normal (a) and homozygous $\delta\beta$ thalassaemia RNA (b). Between 2,000 and 2,500 c.p.m. of α or β cDNA were hybridised to 5×10^{-6} to 1.2×10^{-2} μg of normal 6 to 16S RNA for 4 h, and to 1×10^{-4} to $1 \mu\text{g}$ of $\delta\beta$ thalassaemia 6 to 16S RNA for 24 h to obtain the C_0t values shown. Hybridisation was performed in a 10- μl reaction mixture at pH 7.0 containing (final concentration): 0.02 M sodium phosphate, 0.1% sodium dodecyl sulphate, 0.3 M NaCl and 0.002 M EDTA at 68 °C. The percentage hybridisation was determined after digestion of single-stranded cDNA with micrococcal nuclease: 4- μl samples of each hybridisation mixture were treated with micrococcal nuclease and 4 μl was used as control for recovery in individual experiments. Δ , α cDNA; $\circ$, β cDNA.

Table 1 Amounts of α and β mRNA and percentage β cDNA hybridised

Source of RNA*	α/β mRNA content†	% β cDNA hybridised‡
Normal (8)§	0.9-1.36	90-96
β^+ thalassaemia (6)	4.5-14.3	82-88
$\delta\beta$ thal homozygote	0	18
β^0 Catania --1	2	80
--1	2.4	80
β^0 Ferrara --1	1.8	50
--2	1.78	54
--3	1.43	46
--4	1.04	56
--5	0.4	48

*6 to 16S RNA was isolated by sucrose density gradient centrifugation in all cases from peripheral blood.

† $C_0t_{1/2}$ β cDNA/ $C_0t_{1/2}$ α cDNA. The $C_0t_{1/2}$ is the C_0t at the midpoint between the initial and final hybridisation plateau.

‡The % of β cDNA resistant to micrococcal nuclease digestion at the final hybridisation plateau. The background without added RNA has not been subtracted.

§Number of patients studied is in parentheses.

||Studies performed twice on the same patient.

No β globin synthesis was demonstrable by column chromatographic analysis when peripheral blood samples containing reticulocytes from β^0 Catania, β^0 Ferrara or $\delta\beta$ thalassaemia samples were incubated with ^3H -leucine. A typical chromatographic separation on carboxymethyl cellulose⁴ in a patient from Ferrara is shown in Fig. 1. In contrast to previous studies¹⁸, there is no induction of β globin synthesis 22 d after blood transfusion. Similar results were obtained with β^0 Catania cells and with homozygous $\delta\beta$ thalassaemia cells¹³. When mRNAs from $\delta\beta$, β^0 Catania and β^0 Ferrara patients are added to a Krebs' ascites tumour cell-free system (ref. 19 and R. G. and B. L., unpublished) there is also no detectable synthesis of β globin chains. Only α and γ globin synthesis are present.

Using normal reticulocyte 6 to 16S RNA, the relative content of α and β mRNA measured by hybridisation to purified cDNAs is close to 1.0 (Table 1, Fig. 2a). In addition, greater than 90% of the α and β cDNAs are protected from micro-

coccal nuclease digestion by normal reticulocyte mRNA, indicating that all of the nucleotide sequences in the cDNA are represented in the mRNA. Using 6–16S RNA isolated from the $\delta\beta$ thalassaemia homozygote, the α cDNA hybridises to a saturation plateau of 70% at a relatively low C_{ot} value while at a C_{ot} value 30–40-fold above this, only 18% of the β cDNA is hybridised (Table 1, Fig. 2b). The background of the hybridisation in the absence of added mRNA is 9%. These data indicate that $\delta\beta$ thalassaemia homozygote mRNA contains no detectable δ or β mRNA sequences, and confirms previous studies⁸. In addition, they show that the β cDNA probe used is contaminated with less than 10% α cDNA. The fact that only 70% of the α cDNA is hybridised in a vast excess of $\delta\beta$ thalassaemia mRNA (Fig. 2b) suggests that the α cDNA is contaminated to approximately 20–25% with β cDNA sequences. Additional evidence supporting this is obtained when the α cDNA is hybridised with hydrops fetalis mRNA (containing no α mRNA)⁹; only 20 to 25% of the α cDNA is protected while β cDNA is hybridised to 85% (Fig. 3a).

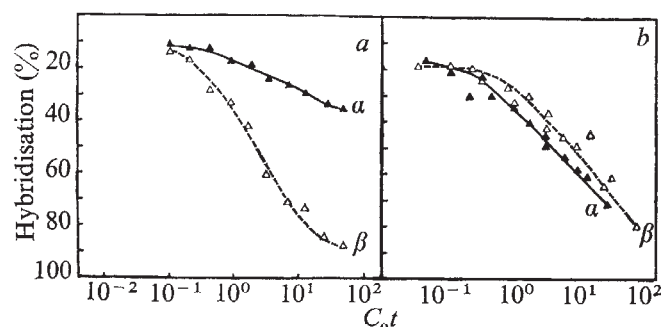


Fig. 3 Hybridisation of α and β cDNA with hydrops fetalis (a) and β^0 Catania RNA (b). 2,000 and 2,500 c.p.m. of α or β cDNA were hybridised to 2×10^{-2} to $10 \mu\text{g}$ of hydrops fetalis 6 to 16S RNA for 4 h and to 1.3×10^{-4} – $6.4 \times 10^{-1} \mu\text{g}$ of β^0 Catania 6–16S RNA for 120 h. The hybridisation conditions and analysis are as described in the legend to Fig. 2. $\blacktriangle$, α cDNA; $\triangle$, β cDNA.

When 6–16S mRNA from β^0 Catania cells is hybridised to α and β cDNAs, a completely different hybridisation curve is obtained. The C_{ot} with the β cDNA is 2- and 2.4-fold greater than that with α cDNA indicating 2- to 2.4-fold greater amounts of α than β globin mRNA sequences are present (Fig. 3b, Table 1). Here, 80% of the β cDNA is protected from micrococcal nuclease digestion using the RNA of this patient. Although it is clear from Fig. 3b that almost all of the β cDNA is hybridised and the curve is continuing to descend, a high enough C_{ot} value was not attained to determine if all of the nucleotide sequences in the β cDNA are present in the β mRNA molecules in the cells of this patient. More extensive studies at higher C_{ot} values in this and other patients with β^0 Catania thalassaemia are required to determine if all of the sequences present in intact β mRNA are present. However, the extent of β cDNA hybridisation is clearly different from that of $\delta\beta$ thalassaemia mRNA. These data are consistent with those reported previously in two patients using rabbit globin cDNA probes⁶ and in two Chinese patients and a β^0 patient of Italian extraction¹¹ suggesting the possibility that there is non-functional β globin mRNA in some patients with β^0 thalassaemia. Since in these^{6,11} and the present studies, less than full-length β cDNA probes are used, even complete hybridisation of the β cDNA would not prove that complete β globin mRNA molecules are present and untranslatable. To do this, studies using full-length cDNA and structural analysis of the β mRNA will be required. The results reported here also do not exclude the possibility that some patients with β^0 thalassaemia from Catania may have

an absence of β mRNA as has been reported in other β^0 patients^{8,12}.

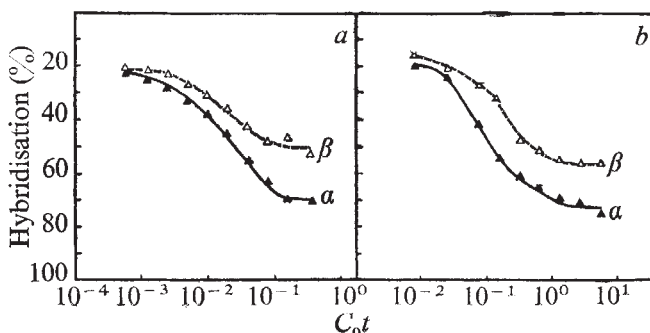
Defect in mRNA from β^0 Ferrara cells

When β^0 Ferrara 6–16S RNA is hybridised to the α and β cDNA probes, a different result is obtained from that with normal, $\delta\beta$ or β^0 Catania mRNA. First, the β cDNA is only hybridised to 46–56% at C_{ot} values even in mRNA excess 10- to 25-fold greater than that necessary to protect the α cDNA maximally (Table 1, Fig. 4). Using cDNA synthesised from HbH mRNA, similar extents of hybridisation were obtained to those using β cDNA, 45–55%. The C_{ot} values are an accurate measure of the relative amounts of α and β mRNA present in a sample of RNA^{6,9,20}. The C_{ot} values for α in Fig. 4 are 2×10^{-2} and 9×10^{-2} respectively. If a small amount of intact β mRNA capable of hybridising to all of the β cDNA is present in β^0 Ferrara mRNA, it must represent less than 5% and 2% of α mRNA since the C_{ot} of any complete β mRNA must be at least 4×10^{-1} and 6 (highest C_{ot} values reached) respectively.

Mixing of two β^0 Ferrara mRNA preparations in different combinations did not increase the percentage hybridisation of β cDNA. By contrast, mixing of β^0 Ferrara mRNA with hydrops fetalis mRNA or normal mRNA leads to over 90% hybridisation of the β cDNA, indicating that no inhibitor of β mRNA hybridisation is present in the β^0 Ferrara mRNA samples. When β^0 Ferrara is hybridised to β cDNA at 78 °C instead of 68 °C, there is a decrease in the amount of cDNA protected from micrococcal nuclease digestion to a background value. The hybridisation of normal reticulocyte mRNA to β cDNA and of normal and β^0 Ferrara mRNA to α cDNA are unaffected by a similar increase in temperature. The lower level of protection of α cDNA by β^0 Ferrara mRNA than by normal mRNA can be explained by contamination of α cDNA by β cDNA sequences. This was confirmed by showing that when hydrops fetalis mRNA containing only β and γ mRNA was added to the Ferrara mRNA, 90% of the α cDNA probe could be protected.

In β^0 Ferrara mRNA, it is clear that different hybridisation results are obtained than with β^+ and β^0 Catania and $\delta\beta$ mRNA. Even in large mRNA excess only 46–56% of the β cDNA is hybridised; only about half of the nucleotide sequences present in β cDNA are represented in β Ferrara RNA in significant amounts. This finding cannot be explained by contamination of the β cDNA probe by α cDNA since only 18% of the β cDNA is hybridised at similar inputs of RNA from $\delta\beta$ thalassaemia cells which also contain α mRNA in normal amounts. In all five β^0 Ferrara patients studied, the defects in β mRNA are similar if not identical since there is no increase in saturation

Fig. 4 Hybridisation of α and β cDNA with β^0 Ferrara-1 RNA (a) and β^0 Ferrara-2 RNA (b). Between 2,000 and 2,500 c.p.m. of α or β cDNA were hybridised to $4 \times 10^{-4} \mu\text{g}$ to $3 \times 10^{-1} \mu\text{g}$ of β^0 Ferrara-2 RNA for 8 h, and to 1.2×10^{-4} – $8 \times 10^{-2} \mu\text{g}$ β^0 Ferrara-1 RNA for 4 h. The hybridisation conditions and analysis were as described in the legend to Fig. 2. $\blacktriangle$, α cDNA; $\triangle$, β cDNA.



hybridisation with β cDNA when mixing of β^0 Ferrara mRNA samples were performed.

The possibility that the β cDNA is hybridising with γ mRNA sequences in β^0 mRNA was studied by comparing the hybridisation of γ cDNA prepared as described previously²¹ with $\delta\beta$ thalassaemia and β^0 Ferrara mRNA. Using both types of mRNA the γ cDNA probe was hybridised at C_{0t} values two- to threefold less than using α cDNA, indicating approximately one-third to one-half as much γ as α mRNA is present in both of these mRNAs (F.R., J.V.O'D., C. Natta and A.B., unpublished). The hybridisation data support cell-free studies indicating that similar amounts of γ globin mRNA are present in β^0 Ferrara and $\delta\beta$ mRNA. The results with $\delta\beta$ mRNA indicate that γ mRNA does not cross hybridise with the β cDNA probe in the conditions of hybridisation, and that the difference in the extent of hybridisation of β cDNA to $\delta\beta$ and β^0 Ferrara mRNA cannot be explained by the presence of γ mRNA.

Several possible mechanisms can explain the 35–45% difference in hybridisation of β^0 Ferrara mRNA and $\delta\beta$ thalassaemia mRNA with β cDNA. First, β mRNA may be completely absent in these cells, but since δ mRNA is present in β^0 Ferrara cells, the partial hybridisation may be due to homology between β cDNA and δ mRNA nucleotide sequences. Since β and δ globin chains differ by only 9 of 144 amino acids, significant homology at the nucleotide level is not unlikely. But the relatively low C_{0t} of hybridisation to β cDNA when compared with hybridisation to α cDNA (Fig. 4) requires a rather large amount of δ mRNA to be present in these cells.

If incomplete or structurally abnormal mRNA existed, the abnormality in β^0 Ferrara mRNA could be either at the 5' or 3' end of the mRNA outside of the nucleotide sequence necessary to encode β globin; the complete nucleotide sequence in mRNA necessary to encode β globin could be intact, although this mRNA might be more labile than normal mRNA and reports of induction of β globin synthesis by transfusion *in vivo* and addition of supernatant fractions *in vitro* would be possible^{18,23}. Regardless of the precise explanation for the limited hybridisation of Ferrara mRNA to β cDNA, the results suggest that the genetic lesion in β^0 Ferrara is different from that in β^0 Catania, β^+ and $\delta\beta$ thalassaemia and is associated with either an absence of β globin mRNA or an abnormal β globin mRNA. The precise nature of the gene defects in the β^0 Catania and β^0 Ferrara and perhaps other forms of β^0 thalassaemia must await analysis of newly synthesised β globin mRNA sequences in the nuclei of erythroid cells in patients with these syndromes.

Deletion of globin gene sequences in $\delta\beta$ thalassaemia

When β cDNA is hybridised to normal DNA approximately 51–56% of the β cDNA is hybridised at similar cDNA:cellular DNA inputs (Table 2). DNA from normal fibroblasts, spleen and white blood cells all give similar levels of hybridisation. When DNA from β^0 Catania cells is hybridised to β cDNA, approximately 50–55% of the β cDNA becomes hybridised. These results indicate that there is no detectable deletion of

Table 2 Hybridisation of α and β cDNA to cellular DNA

Source of DNA	Percent hybridisation*			
	α cDNA C_{0t} : 4,320	α cDNA C_{0t} : 8,640	β cDNA C_{0t} : 4,320	β cDNA C_{0t} : 8,640
Normal -1	43	39	51	51
Normal -2	47	49	54	56
Normal -3	47	46	51	51
HPFH homozygote	35	36	23	22
$\delta\beta$ homozygote	37	41	32	29
$\delta\beta$ heterozygote -1	44	44	38	41
$\delta\beta$ heterozygote -2	40	43	41	43
β^0 Catania -1	47	46	55	50
β^0 Catania -2	45	43	56	54
Hydrops fetalis	21	20	53	53

*In each cDNA-DNA hybridisation, 3.3×10^{-7} as much cDNA as cellular DNA is used. Forty-five μ l aliquots containing 0.135 mg of DNA and 630 c.p.m. (0.045 ng) of either α or β cDNA were mixed in 0.12 M sodium phosphate (pH 6.8) and 0.4% sodium dodecyl sulphate in 100- μ l capillary pipettes, heated in a boiling water bath for 15 min and incubated at 68 °C for varying times in order to attain the C_{0t} values shown. The cDNA eluted from hydroxylapatite at 68 °C by 0.4 M sodium phosphate is expressed as a percentage of cDNA eluted by 0.12 M plus 0.4 M sodium phosphate successively. The values obtained at different C_{0t} values are shown to indicate a saturation plateau has been reached.

Alternatively, the RNA sequences hybridising to β cDNA may represent Lepore-like globin mRNA in the Ferrara RNA, and the hybridisation to β cDNA due to homology with the β -like sequences in this Lepore-like chain. Or third, there may be a partial deletion of β globin mRNA sequences at the 3' end of Ferrara mRNA. In addition to 50–100 polyadenylate residues at the 3' end of human globin mRNA²², studies of abnormal elongated human globin mutants suggest that there is an untranslated nucleotide sequence of approximately 100 nucleotides at this end¹. Since β globin cDNA contains approximately 400 nucleotides and primarily sequences synthesised from the 3' end of mRNA, a deletion at the 3' end of the Ferrara mRNA might result in the findings reported here. Lastly, the presence of normal amounts of a structurally abnormal mRNA with either increased lability or considerable secondary structure must be considered. Such an abnormal RNA might not be fully hybridised to β cDNA. Either cross hybridisation of β cDNA to δ mRNA sequences or an abnormal β mRNA could explain the lability of the β^0 Ferrara mRNA- β cDNA hybrid.

β or β -like genes in β^0 thalassaemia. In contrast, when HPFH DNA is mixed with β cDNA only 22–23% of the β cDNA is hybridised at saturation (Table 2). Using DNA from a $\delta\beta$ thalassaemia homozygote, only 32 and 29% of the β cDNA is hybridised, somewhat higher than that using HPFH DNA (Table 2). Similarly, DNA from heterozygotes for $\delta\beta$ thalassaemia show decreased hybridisation to the β cDNA probe to 41 and 43%, respectively (Table 2). To determine if the deletions in HPFH and $\delta\beta$ thalassaemia DNA are overlapping, 0.112 mg of homozygous $\delta\beta$ thalassaemia DNA and 0.113 mg of HPFH DNA were mixed and annealed with 0.045 ng β cDNA to a C_{0t} of 8,640; 37% of the β cDNA was protected. 0.225 mg of normal DNA gave 59% hybridisation while the same amount of HPFH DNA gave 24% hybridisation with the same input of β cDNA. The data are consistent with overlapping gene deletions in HPFH and $\delta\beta$ thalassaemia DNA with a larger deletion in HPFH DNA.

When α cDNA is hybridised to cellular DNA from normal, HPFH, $\delta\beta$ and β^0 thalassaemia cells at similar inputs of cDNA

and cellular DNA, relatively similar amounts of hybridisation of the α cDNA are obtained (Table 2). These values indicate that normal, HPFH, and β^0 and $\delta\beta$ thalassaemia DNA all contain similar numbers of α globin genes and serve as a useful internal control of the cellular DNA : cDNA input. There is 5–10% less hybridisation of α cDNA with HPFH, and 3–7% less hybridisation of α cDNA with $\delta\beta$ thalassaemia DNA from the homozygote than with similar inputs of normal or β^0 thalassaemia DNA (Table 2). This is consistent with β cDNA sequences in the α cDNA probe which cannot be hybridised because of the deletion of β -like cellular DNA sequences. The somewhat higher level of hybridisation of normal DNA to cDNA than to α cDNA (51–56% compared to 39–49%) confirms previous data⁹.

Studies to date indicate that in homozygous β^+ thalassaemia⁹, β^0 thalassaemia¹² and in a $\delta\beta/\beta^0$ double heterozygote¹⁰ there is no detectable deletion of globin structural genes. In contrast, deletion of globin structural genes has been reported in HPFH homozygotes^{15,24}. Our results confirm deletion of β -like globin DNA nucleotide sequences in HPFH DNA (Table 2). Less than half as much hybridisation of β cDNA occurs with HPFH DNA as with similar input of normal DNA. The nature of the sequences in HPFH DNA which hybridise to β cDNA is not yet defined; they may represent incompletely deleted δ and/or β structural gene loci, embryonic β -like genes or cross hybridisation of α gene sequences to the α cDNA contaminating the β cDNA probe.

Deletion of β structural gene sequences has recently been observed by others (S. Ottolenghi *et al.*, unpublished) in patients homozygous for $\delta\beta$ thalassaemia. We have confirmed the deletion of β -like gene material in one of these homozygotes. Further, we demonstrate detectable deletions of β -like gene material in heterozygotes for $\delta\beta$ thalassaemia (Table 2). Our methodology seems to be more sensitive in detecting gene deletions in $\delta\beta$ heterozygotes than hybridisation in vast DNA excess or vast cDNA excess, since such a deletion was not detected by others using these methods¹⁰. These data are consistent with at least partial deletion of β or δ globin structural genes or parts of both in this disorder. The deletion of structural globin gene sequences in $\delta\beta$ thalassaemia DNA may, in addition, be less than that in HPFH DNA. In both HPFH and homozygous $\delta\beta$ thalassaemia, the only haemoglobin produced is foetal haemoglobin, HbF. In HPFH, sufficient γ globin is produced to maintain normal haemoglobin levels, while in $\delta\beta$ thalassaemia, γ globin production does not compensate and there are cellular abnormalities typical of thalassaemia and consequent haemolytic anaemia. Regulation of γ globin synthesis at the gene level has been postulated to involve regulatory nucleotide sequences adjacent to the δ structural genes²⁵. It may be speculated that deletion of these sequences in HPFH permits maximal expression of γ globin genes even in adult life. The differences in hybridisation between HPFH and $\delta\beta$ thalassaemia DNA with β cDNA reported here could reflect the presence of residual sequences in $\delta\beta$ thalassaemia DNA with a suppressive effect on γ globin production. The residual sequences present in $\delta\beta$ thalassaemia and absent in HPFH DNA may generate a gene product which suppresses γ gene activity. Alternatively, no such diffusible gene product acting *in cis* would be required if the sequences we are detecting in $\delta\beta$ thalassaemia which are absent in HPFH are adjacent to the γ globin genes and are involved in the normal suppression of γ globin gene activity in adult life. These speculations do not exclude the possibility that other genetic interactions may control γ gene expression.

Recently, a patient with β^0 thalassaemia has been described in whom there is absence of β mRNA but no apparent deletion of β -like genes¹². In the present and previous studies^{9,12} of patients with β^0 and β^+ thalassaemia who have intact δ globin loci, it is possible that cross hybridisation between β cDNA and δ gene sequences can obscure the detection of small deletions in β structural genes. In addition, the use of less than complete length β cDNAs in all of the studies reported to date

of gene deletion in β and $\delta\beta$ thalassaemia make it possible that deletions of β -like sequences in DNA at the end of β or δ genes homologous to the 5' end of β globin mRNA may exist and remain undetected in these syndromes.

The common denominator of the β thalassaemia syndromes is decreased β globin synthesis with inadequate γ globin compensation. The decrease in β globin production in β^+ thalassaemia is associated with decreased β globin mRNA and is due to either a defect in regulatory genes controlling β structural gene expression or the production of a structurally abnormal β gene product which is partially destroyed during RNA processing in the nucleus. In one patient with β^0 thalassaemia, absent β mRNA is associated with no detectable deletion of β globin genes¹². On the other hand, as demonstrated in this paper, the absent δ and β mRNA in $\delta\beta$ thalassaemia is associated with deletion of δ and/or β gene material. In some β^0 thalassaemia patients^{6,11} including one from Catania described here there are significant amounts of incomplete or complete β mRNA sequences in cells which are untranslated and may reflect a small abnormal nucleotide sequence in the β globin gene. This report provides evidence that β^0 Ferrara may differ from other forms of β^0 thalassaemia described to date and is associated with either abnormal or absent β mRNA. Thus, the β thalassaemia syndromes represent a heterogeneous group of disorders at the genetic level, all of which lead to decreased β globin production.

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Enzyme evolution in a microbial community growing on the herbicide Dalapon

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*A seven-membered microbial community capable of utilising the herbicide Dalapon has been isolated by continuous-flow enrichment culture. The composition of this community has remained remarkably stable over thousands of hours in a Dalapon-limited chemostat. During this period, however, one member of the community, *Pseudomonas putida*, acquired the ability to grow on Dalapon through the evolution of an extant dehalogenase.*

In most natural environments different populations of micro-organisms grow in close proximity to each other and may utilise similar substrates present in the ecosystem. One likely consequence of this is that various nutritional and physical interactions will develop between the populations leading to the evolution of complex, mutually dependent and possibly highly stable microbial communities. It is reasonable to postulate that interacting microbial communities are common in nature; however, very few microbial associations of this type have been defined, principally because inappropriate enrichment and selection techniques have been used to isolate them from natural environments.

The aims of the work reported here was to use suitable continuous-flow culture enrichment procedures^{1,2} to isolate a microbial community which could grow on the herbicide 2,2'-dichloropropionic acid (Dalapon, Dow Chemical Company) as its sole source of carbon and energy. Dalapon is a widely used herbicide which is effective against monocotyledons and is known to undergo microbial degradation³. Such a compound was selected because it was argued that mixed populations possessing diverse metabolic capabilities are more likely to be able to metabolise recalcitrant or semi-recalcitrant compounds than are populations composed of a single species. In addition, it seems to be the general case that so called 'environmentally foreign' compounds are degraded much more rapidly in natural environments than they are by monocultures of micro-organisms grown in similar conditions in the laboratory. Such observations provide circumstantial evidence for cooperative metabolic attack by a microbial community.

Characterisation of isolated microbial community

Figure 1 describes the structure of the microbial community which has been isolated on several occasions from four different soil sources, two of which had been pretreated with Dalapon. Initially, after three weeks growth in the chemostat enrichment system, the mixed culture consisted of seven different microorganisms which could be divided into two groups. The first group comprised the primary utilisers which, in monoculture, were able to grow on Dalapon as the only carbon and energy source. In all four microbial communities originally isolated, three primary utilisers were

found; two were Gram-negative, motile rod bacteria, one of which (P1) was identified as a *Pseudomonas* species of Shewan's group II (ref. 4) the other (P2) remains unidentified. The third Dalapon utiliser was a filamentous fungus, *Trichoderma viride* (P4). The other group contained the secondary utilisers which were unable to grow on Dalapon in monoculture in any growth conditions. These secondary organisms must have been growing on metabolites which were produced directly from the primary catabolism of Dalapon, or metabolites excreted by the growing primary utilisers, or on compounds freed into the growth medium as a consequence of the death and lysis of primary and secondary utilisers. The non-Dalapon utilisers were three bacteria—a *Flavobacterium* species (S1), *Pseudomonas putida* (S3), and an unidentified pseudomonad (S4)—and a pink budding yeast (S5). The quantitative species composition remained very stable with changing growth conditions with the exception of the two major primary Dalapon utilisers; at low dilution rates in the chemostat (less than 0.25 h⁻¹) the bacterium P2 was the dominant primary organism whereas at high dilution rates (greater than 0.45 h⁻¹) *Pseudomonas* species P1 dominated.

Stability of the community

The yeast was only loosely associated with the microbial community and was eliminated from the enrichment chemostat the first time the dilution rate was raised above 0.2 h⁻¹.

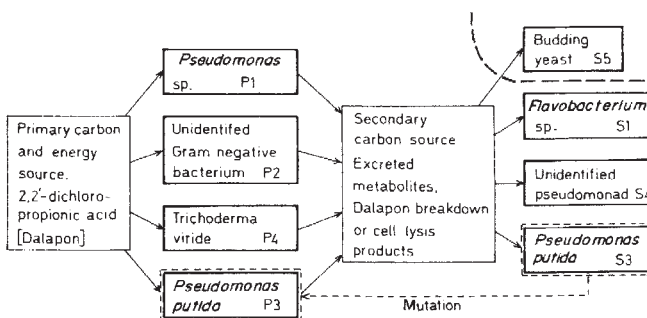


Fig. 1 The structure of the microbial community growing on 2,2'-dichloropropionic acid. The chemostat enrichment cultures had a volume of 0.5 l and were operated at a dilution rate (D) of 0.015 h⁻¹. The growth medium consisted of (g l⁻¹): K₂HPO₄, 1.5; KH₂PO₄, 0.5; (NH₄)₂SO₄, 0.5; MgSO₄·7H₂O, 0.2; Dalapon, mixed Na and Mg salts (Dow Chemical Company, UK) 1.65; pH 7.3. Several enrichments were set up using as inocula, *a*, culture effluent from a soil column containing a sandy loam (pH 4.8) which had been pretreated with Dalapon (the column had been perfused with Dalapon-containing minimal medium for several weeks before some of the culture effluent was used as the inoculum) and *b*, soil, either sandy loam or sandy chalk, which had or had not been pretreated with Dalapon, added directly to the chemostat. The enrichment cultures were monitored for biomass (culture absorbance and dry weight), species composition, pH and residual Dalapon concentration (determined by measuring chloride ions in solution by modification of Mohr's method⁸).

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The remaining six-membered association was exceedingly stable, however. One of the isolated communities was cultured for over 7,500 h at constant growth rate and environmental conditions with no qualitative change in the species composition. A second six-membered community has been continuously grown in a Dalapon-limited chemostat for over 13,500 h, which corresponds to approximately 3,700 generations. Throughout this period, and in spite of repeated environmental perturbations and changes in the specific growth rate, only one significant change in the community's character has been detected and this is described later. This particular community has been subjected to regular changes in dilution rate, such that seven continuous cycles of increasing and decreasing growth rate have been completed.

In the first cycle (Fig. 2) the dilution rate was increased by small increments and steady-state cultures were established at each value. At growth rates below $D=0.25 \text{ h}^{-1}$ the culture pH remained approximately constant at 3.3. The reduced pH in the poorly buffered growth medium and in the absence of fermenter pH control was due to the complete breakdown of the available Dalapon and the concomitant production of HCl. With a further slight increase in the dilution rate there was a marked rise in the culture pH to 6.1. This shift in pH was simultaneously accompanied by an increase in the unused Dalapon concentration and a decrease in the culture biomass. Further increases in the dilution rate, up to a maximum of 1.1 h^{-1} , caused further increases in the pH up to a maximum of 6.9. When the dilution rate was gradually decreased to complete the first cycle of dilution rate changes, a reverse hysteresis-like pattern of pH change was observed with the rapid phase of pH decline occurring at dilution rates between $D=0.19$ and 0.17 h^{-1} .

With successive cycles of changing dilution rate, the rate of pH change decreased until, by the seventh cycle, the pH change took place gradually over almost all the dilution rate range examined; moreover, in these later cycles the culture pH did not fall to the original low values. These observations indicated that to some extent the microbial community gradually stabilised, without qualitative changes in the species composition of the association, to dampen and minimise the environmental changes which occurred with changes in the dilution rate. The overall effect was to reduce the potentially deleterious influence of rapid pH fluxes.

During the increasing phase of the first dilution rate cycle (Fig. 2), the sudden pH shift was accompanied by an immediate development of heavy growth on the walls throughout the chemostat vessel. When the dilution rate was decreased the wall growth disappeared once the pH values had dropped to ~ 4.0 . In later cycles the appearance of wall growth was much more gradual, probably because the change in pH was correspondingly less rapid. In all these latter cycles, however, heavy wall growth became re-established when the pH had risen to greater than 5.0. The composition of the wall growth was complex, but it contained all the individual species of the microbial community originally present in free suspension. The attached surface growth was dominated by the mycelia of *Trichoderma viride*, however, and this contrasted markedly with the microflora in suspension at the same dilution rates, from which the fungus was virtually absent. At the lower dilution rates, and hence at low pH values, hyphae of *T. viride* were dispersed homogeneously in the culture. The development of wall growth prevented washout of the culture at dilution rates exceeding the maximum specific growth rate of the community and effectively preserved the integrity of the community in such circumstances. Submerged liquid batch and agar plate cultures of *T. viride* growing on Dalapon proved that the fungus readily metabolised the herbicide and grew vigorously at low pH values whereas at $\text{pH}>6.0$

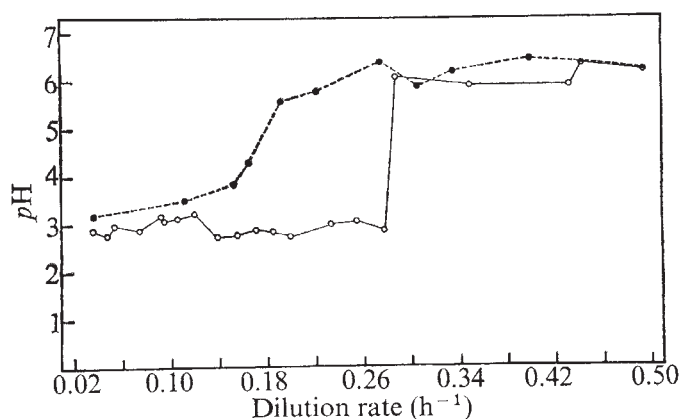


Fig. 2 The change in steady-state culture pH for the six-membered microbial community growing in a Dalapon-limited chemostat during the first cycle of increasing and decreasing the dilution rate. ○, Steady-state pH values in the increasing dilution rate phase; ●, pH values during the decreasing dilution rate phase. Heavy wall growth dominated by *T. viride* was established at growth rates in excess of $D = 0.28 \text{ h}^{-1}$, during the increasing dilution rate phase, and had disappeared between $D = 0.19$ and 0.17 h^{-1} during the decreasing stage.

growth was extremely sparse. Thus fungal growth on the internal surface of the chemostat seemed to be a response to unfavourable conditions, namely high pH values, which developed at high dilution rates, and an attempt to create a favourable microenvironment within the chemostat where low pH conditions could be established and maintained.

New primary Dalapon utiliser

The significant change in the nature of the microbial community, first noticed after 2,900 h of continuous growth, was the appearance of a fourth primary Dalapon utiliser, subsequently identified as *P. putida* (P3) and identical in all other respects to the secondary utiliser, *P. putida* S3. It seemed possible that P3 had arisen as a mutation of S3 during the prolonged period of growth under Dalapon-limited conditions. The evolvant was retained within the community, together with its parent S3, and did not seem to unbalance the system. It is interesting that the selection of another primary utiliser should have occurred in spite of the presence of the three original primary utilisers and the inevitable increase in competition for the growth-limiting nutrient. Indeed the presence of three or four primary utilisers apparently contradicts the kinetic theory of enrichment cultures^{5,6} which predicts that the organism with the greatest affinity for the limiting nutrient (that is, the lowest saturation constant) or the highest maximum specific growth rate will be the most successful in competing with others. In open systems, such as continuous-flow cultures, the most competitive organism ought to cause the elimination of the less competitive population. Our observations strongly indicate that several, so far unidentified, interactions occur between the individual populations of the whole community and these serve to stabilise free competition between the primary utilisers. The catabolism of Dalapon by hydrolysis to pyruvic acid and free chloride occurs as a result of the activity of the enzyme dehalogenase⁷. Thus *P. putida* P3 could have arisen either by the acquisition of Dalapon-metabolising capability by plasmid transfer from one of the original primary utilisers, or, by the selection of a mutant having a modified dehalogenase activity enabling it to metabolise Dalapon.

The latter hypothesis has been verified in monoculture chemostat studies. *P. putida* S3, isolated from the microbial community and purified could not grow on Dalapon in

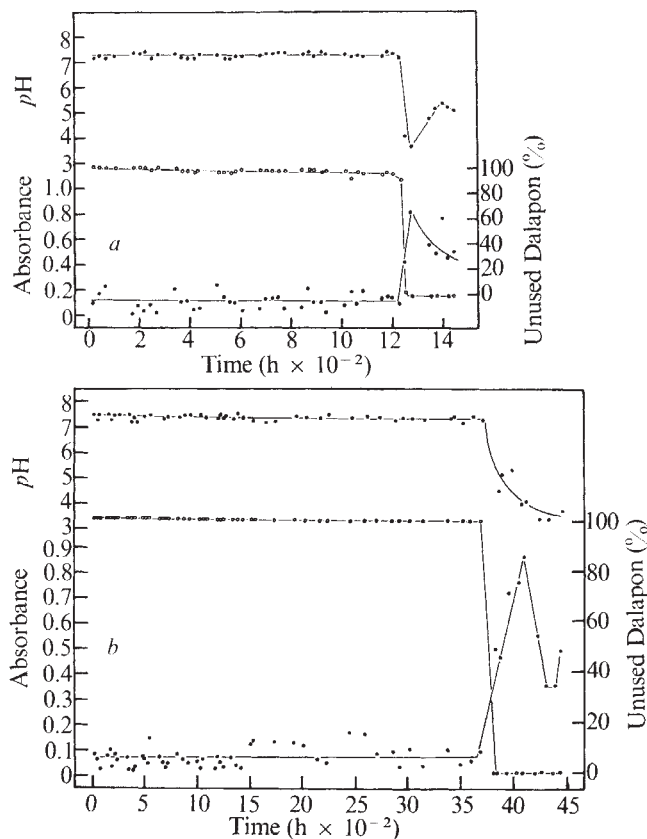


Fig. 3 *a* and *b*, Two experiments demonstrating the evolution of Dalapon-metabolising *P. putida* P3 from the non-Dalapon-metabolising *P. putida* S3. See text for details. Lower ●, culture absorbance (E_{625}); ○, unused Dalapon as a percentage of the concentration in the inflowing medium. Top ●, culture pH.

a range of environmental conditions. A culture of *P. putida* S3, grown on sodium propionate, was inoculated into a chemostat containing a minimal growth medium with the carbon source as the growth-limiting substrate and operating at a dilution rate of 0.05 h⁻¹. The carbon source was a mixture of sodium propionate (0.07 g carbon l⁻¹) and Dalapon (0.35 g carbon l⁻¹) which gave a carbon ratio of 1 : 5. S3 was initially able to grow only on the propionate carbon and thus could be retained in the open culture system and at the same time be continuously exposed to a non-utilisable carbon source. Accordingly evolvents with the capacity to utilise the additional carbon source might be expected to have a considerable selective advantage if they could acquire the capacity to metabolise Dalapon.

Figure 3*a* and *b* shows the results of two such evolution experiments. In the first experiment (Fig. 3*a*) a steady-state culture of strain S3, growing solely on propionate was established with a culture absorbance of approximately 0.1, a situation that was maintained for just over 1,200 h of continuous growth in constant environmental conditions. During the first stage of the experiment the culture pH remained constant at its initial value of 7.4, suggesting that no Dalapon was being metabolised. This conclusion was confirmed by measuring free chloride in the culture and calculating the percentage of Dalapon which had been metabolised. After 250 h of growth a small number of Dalapon-metabolising colonies were detected on dilution plates. Initially the detection of such colonies was variable but with time their frequency of detection increased until they were found in every sample taken from the chemostat. The presence of Dalapon-metabolising individuals within the population did not immediately result in their competitive selection and displacement of the S3 population.

After 1,200 h of growth, however, such an event did occur and was manifest as an approximately fivefold increase in the culture absorbance, a result which would be anticipated if the evolvent population were able to utilise the additional carbon source. Metabolism of Dalapon was indicated by a concomitant decrease in culture pH and an increase in free chloride in the culture. Within 30 h the evolvent P3 succeeded in establishing a new steady-state culture and completely displacing the original S3 strain. In the new steady state the Dalapon concentration in the chemostat was reduced to zero. A similar sequence of events has been observed in five other experiments, the only variation being the time at which the Dalapon-metabolising population managed to replace the parent strain. In a second experiment (Fig. 3*b*) for example, the mutational event leading to the selection of the P3 strain occurred much later, after 3,750 h of continuous growth, in conditions identical to those of the first experiment.

Preliminary dehalogenase analyses have shown that during the first 3,750 h of growth of *P. putida* S3 (Fig. 3*b*) there was no dehalogenase activity with a specificity towards Dalapon. After the mutational event and the establishment of the new steady-state culture however, Dalapon-specific dehalogenase activity was detected (E. Senior, C. Larvin, J. H. Slater and A. T. Bull, unpublished). In addition, we have obtained evidence which shows that the dehalogenase originally possessed by *P. putida* S3 had a specificity towards 2-monochloropropionic acid and that the evolvent P3 has a markedly increased specific activity of the 2-monochloropropionic acid-specific dehalogenase (J. H. Slater, D. E. Sanders and A. T. Bull, unpublished). Growth experiments have indicated that *P. putida* S3 will grow slowly on 2-monochloropropionic acid as its sole carbon source, whereas P3 grows vigorously on this substrate.

Enzyme evolution

Experimental enzyme evolution is an area that has excited growing interest over the past decade and elegant demonstrations of the evolution of enzymes with new specificities and activities have been provided by studies of the aliphatic amidases of *P. aeruginosa*⁷ and the pentitol dehydrogenases of *Klebsiella aerogenes*⁸. The chemostat offers considerable scope for investigating the evolutionary potentialities of microorganisms, principally because it enables the imposition of precise selection pressures on a population. The putative evolution of *P. putida* dehalogenase is a further vindication of this experimental approach and it is worth emphasising that it was achieved without the intervention of specific mutagens. Moreover, we would like to emphasise the value of mixed microbial populations in enzyme evolution studies in two important respects. First, within such a mixed system multiple selective pressures can operate which are analogous to those occurring in real ecosystems. Second, in the microbial community we have begun to analyse it is clear that the biochemical and physical nature of the whole system provides a permissive environment for metabolic evolution. In particular, the secondary species, by virtue of the fact that they are continuously supplied with assimilable substrates by the activity of the primary utilisers, are retained within the community and thus are continuously exposed to a non-metabolisable compound. Indeed it is clear that for enzyme evolution experiments in monoculture a metabolisable 'carrying' substrate has to be provided to ensure that the single species population continues to grow and be exposed to the non-metabolisable substrate for long periods. We are now attempting to elucidate the precise nature of the dehalogenase in the parent *P. putida* and its relationship to the evolvent strains and to explore the extent to which the catalytic activity of this enzyme can be modified to deal with different chlorinated aliphatic

acids. E.S. thanks the SRC for a research studentship. We thank the Dow Chemical Company for supplying Dalapon; A. Gallenkamp and Co. Ltd, for assistance with fermentation systems and Professor H. Veldkamp and Dr W. Harder for discussions.

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Partial purification of rabbit aorta contracting substance-releasing factor and inhibition of its activity by anti-inflammatory steroids

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Rabbit aorta contracting substance-releasing factor (RCS-RF) is found in perfusates from guinea pig lungs during anaphylaxis. It has been identified as a small peptide which releases arachidonic acid from lung tissue, thus generating prostaglandin endoperoxides and thromboxanes and causing bronchoconstriction. Anti-inflammatory steroids block the release of arachidonate by RCS-RF. In this activity their relative potency is very similar to their relative anti-inflammatory potency, suggesting that the two actions may be related.

PIPER and Vane¹ detected the release of additional substances into the perfusate during anaphylaxis in isolated lungs from sensitised guinea pigs. Because of its activity they called one "rabbit aorta contracting substance" or "RCS". RCS was unstable at room temperature and its release was blocked by aspirin-like drugs. RCS has now been identified as a mixture of the prostaglandin cyclo-endoperoxides (PGG₂ and PGH₂) and thromboxane A₂ (TXA₂)^{2,3}. The RCS activity is mainly due to TXA₂. Piper and Vane also found that after the RCS activity of the perfusate declined, another much more stable substance could be detected. This was called "RCS-releasing factor" or RCS-RF because effluent containing RCS-RF injected into the pulmonary artery of perfused lungs from unsensitised guinea pigs induced a release of "RCS". We have now isolated and partially purified RCS-RF, which seems to be a small peptide. RCS-RF stimulates the generation of PG endoperoxides and TXA₂ probably by releasing the precursor arachidonic acid from lung tissue. Steroid anti-inflammatory drugs block this releasing action and thus prevent the generation of TXA₂, whereas non-steroid anti-inflammatory drugs block the generation of TXA₂ by a direct effect on the prostaglandin cyclo-oxygenase enzyme. The rank order of nine steroid drugs as inhibitors of RCS-RF activity correlates well with their rank order as anti-inflammatory substances.

Isolation, partial purification and assay

The assay procedure for RCS-RF and SRS-A like activity is described in the legend to Fig. 1. Lungs were removed from male guinea pigs (200–300 g) which had been sensitised

to ovalbumin (100 mg subcutaneously and 100 mg intraperitoneally) 2–4 weeks earlier. The lungs were perfused through the pulmonary artery with Krebs' solution at 10 ml min⁻¹ as previously described¹. Anaphylactic shock was induced by injection into the pulmonary artery of 10 mg ovalbumin and the perfusate was collected for 10 min (about 100 ml). Effluent from 5–10 sensitised lungs was pooled, centrifuged (5,000g for 30 min) to remove cells, filtered and stirred for 30 min with 30 g l⁻¹ Amberlite XAD-2 (BDH) to adsorb the RCS-RF activity. After filtering under vacuum to remove the Krebs' solution the Amberlite XAD-2 was washed with small volumes of distilled water,

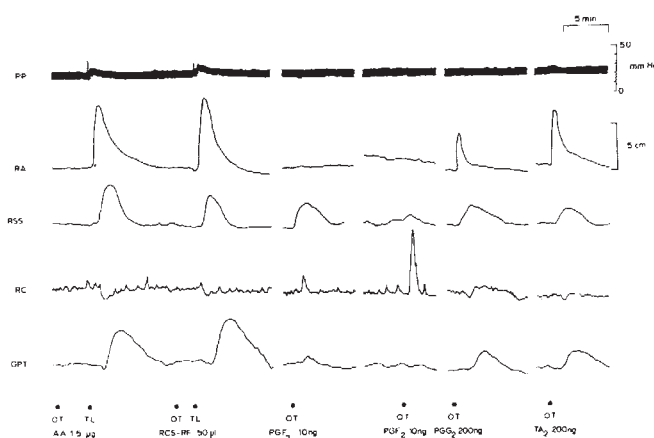


Fig. 1 For bioassay of RCS-RF the effluent from unsensitised lungs was allowed to superfuse a rabbit aortic strip, a rat fundic strip, a rat colon and guinea pig trachea in cascade¹. Sensitivity and selectivity of the tissues were increased by an infusion into the lung effluent of "combined antagonists"¹ to prevent the actions of histamine, acetylcholine, catecholamines and 5-hydroxytryptamine as well as indomethacin (1 µg ml⁻¹) to prevent endogenous synthesis of prostaglandins by the tissues⁴. SRS-A-like activity of samples was estimated using the isolated guinea pig ileum⁵ preparation, hyoscine hydrobromide (10⁻⁷ g ml⁻¹) and, where appropriate, mepyramine maleate (10⁻⁷ g ml⁻¹) was added to the organ bath to prevent spontaneous activity and render the preparation insensitive to histamine⁵. PP, Pulmonary artery perfusion pressure; RA, rabbit aortic strip; RSS, rat (fundic) stomach strip; RC, rat colon; GPT, guinea pig trachea; OT, injection directly over the tissues; TL, injection into the pulmonary artery; AA, arachidonic acid.

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Table 1 Partial purification of RCS-RF

Extraction procedure	Weight of sample	RCS-RF-like activity (Total units)	Yield (%)	Specific activity (U mg ⁻¹)	Purification factor
None. Original effluent lyophilised*	6.64 g	3,534.23	100	0.53	0
Amberlite XAD-2 extraction	245.6 mg	1,473.77	41.70	6.06	11.43
Diethyl ether extraction	236.9 mg	1,208.71	34.20	5.10	9.62
Paper chromatography	0.22 mg	692.12	19.58	3,146.00	5,935.84

The extraction efficiencies vary from experiment to experiment and the figures quoted in this table should only be regarded as a guide.

*Starting material was 570 ml effluent.

and the RCS-RF recovered from the resin with 40–50% yields (see Table 1) by washing with 1.5 l ethanol-water (80:20, v/v). The ethanol-water extract was evaporated *in vacuo* in a rotary film evaporator, and the dried residue was washed three times with dry diethyl ether to remove lipids (thin-layer chromatography of the ether washings revealed substantial quantities of fatty acids, prostaglandins and some phosphatides). After ether washing the residue was dissolved in a small aliquot of distilled water: this preparation, referred to as the “crude fraction”, possesses potent RCS-RF activity and is stable for at least 6 weeks when kept frozen.

For further purification, the crude fraction was streaked on to a silica gel-impregnated paper chromatogram (Whatman SG81) which was developed by ascending chromatography in ammonia-*n*-propanol-water (30:60:10, v/v). After developing to a distance of 20 cm, the chromatogram was removed, dried and cut into 1-cm horizontal sections. There were eluted with small volumes of distilled water and the biological activity assayed.

About 80% of the RCS-RF activity was associated with a single band of R_f 0.7 (see Fig. 2). A small amount of RCS-RF activity was also recovered from a zone running ahead of the major peak and sometimes small amounts were detected close to the origin (see Fig. 2). In addition to the RCS-RF activity, there were several zones containing activity which contracted the guinea pig ileum, indicating SRS-A like activity. One of these corresponded to the bulk of the RCS-RF activity, and others to the minor RCS-RF zones. We regarded the major zone of releasing activity as “RCS-RF” and referred to this fraction as the “partially purified” fraction. The biological activity of the partially purified fraction is shown in Fig. 1. Partially purified RCS-RF has no “direct” effect over the smooth muscle organs but when injected into isolated perfused lungs from guinea pigs it provoked an immediate increase in perfusion pressure, a bronchoconstriction (not shown), and a release of RCS; prostaglandins E or $F_{2\alpha}$ were seldom detected.

The partially purified fraction was stable for several weeks when kept frozen. The releasing activity of RCS-RF was potentiated three- to fivefold by concomitant injections of 10 μ g of an inhibitor of carboxydipeptidase activity BPP-5A (ref. 6). We defined “1 unit” (U) of RCS-RF as that amount which when injected into the lung released the same amount of RCS-activity (TXA₂) as 1 μ g of arachidonic acid.

Physicochemical properties

RCS-RF is insoluble in ether, chloroform, ethyl acetate, acetone and ethanol but is more soluble in methanol (about 70% of its water solubility). It is destroyed by drastic pH changes, being more susceptible to acid rather than alkaline pH values. For example, a 1-h incubation with 1 N HCl completely inactivated RCS-RF but a 1-h incubation with 10 N NaOH still left about 20% of the activity. RCS-RF loses 75% activity after boiling for 10 min, but was unaffected by boiling for 1 min. Passage through a dialysis

membrane suggests a small molecule of <5,000 molecular weight.

The activity of RCS-RF is not reduced by incubation with phospholipase A, C or D (Sigma) (100 μ g enzyme per ml) but was reduced slightly (~15%) by incubation with trypsin (Sigma) (500 μ g enzyme per ml). In two out of four experiments, incubation with arylsulphatase (Sigma) (500 μ g ml⁻¹) produced 50–60% inactivation; SRS-A is also inactivated by this enzyme. Incubation with carboxypeptidase B (Sigma) (500 μ g enzyme per ml for 1 h at 37 °C) or aminopeptidase M (Sigma) (100 μ g enzyme per ml for 1 h at 37 °C) resulted in an 80–85%, or complete inactivation (respectively), strongly suggesting that RCS-RF is a peptide. When a freshly developed paper chromatogram was sprayed with ninhydrin reagent, which reacts with α -amino acyl groups of amino acids, the zone containing RCS-RF was only one of several ninhydrin-positive areas. RCS-RF was not found in the perfusate of non-sensitised lungs challenged with antigen and, unlike RCS, was not released by mechanical trauma. The limited physicochemical data obtained so far are compatible with RCS-RF being a peptide of less than 10 amino acids.

Effects of inhibitors

The release from sensitised lungs of RCS-RF by antigen was not prevented by aspirin (200 μ g ml⁻¹), in the perfusing fluid, indomethacin (2 μ g ml⁻¹), dexamethasone (2 μ g ml⁻¹), disodium cromoglycate (20 μ g ml⁻¹), colchicine (5 μ g ml⁻¹), diethyl carbamazepine (1 mg ml⁻¹), or mepacrine (20 μ g ml⁻¹). Aspirin, indomethacin, mepacrine and dexamethasone (same concentrations) all blocked the release of TXA₂ from the

Fig. 2 Biologically active material recovered from the paper chromatogram. Top panel RCS-RF-like activity assayed as shown in Fig. 1; bottom panel SRS-A-like activity, assayed as described in Fig. 1.

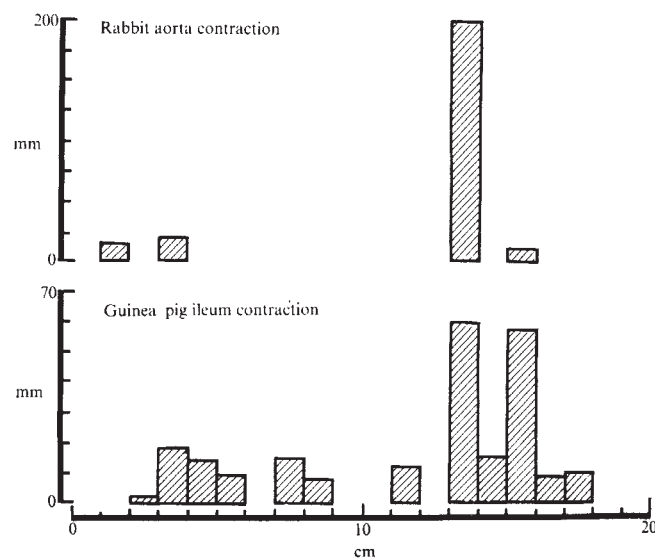


Table 2 ID₅₀ Values for some anti-inflammatory steroids

Steroid	ID ₅₀ (nmol min ⁻¹)	Inhibition (%) of arachidonic acid at ID ₅₀	Relative (molar) potency	Relative anti-inflammatory potency*
Dexamethasone	34.4	— 0.3	35.1	25.0
Betamethasone	36.5	— 7.3	33.1	25.0
Triamcinolone	106.1	+ 31.1	11.4	~5.0
Fludrocortisone	133.0	— 21.9	9.1	10.0
Prednisolone	177.1	— 0.1	6.8	4.0
Prednisone	185.8	— 2.3	6.5	4.0
Corticosterone	968.9	— 32.84	1.3	0.35
Cortisone	1,206.8	+ 9.12	1.0	0.8
Hydrocortisone (cortisol)	1,207.0	— 11.9	1.0	1.0

The following drugs were used: dexamethasone sodium phosphate (Decadron, Merck Sharp and Dohme) or dexamethasone (Sigma); betamethasone (Betsolan, Glaxo); triamcinolone acetonide (Kenalog, Squibb); fludrocortisone (Sigma); prednisolone acetate (Deltastab, Boots); prednisone (Koch-Light); corticosterone (Sigma); cortisone (Sigma); and hydrocortisone sodium succinate (Efcortelan, Glaxo). All steroids were infused for 15 min, and the amounts indicated are calculated as the base. Apart from the soluble esters, steroids were dissolved in ethanol and diluted 9:1 (v/v) with 0.9% saline. Each result is the mean of three or four observations.

*Data taken from ref. 8.

lung provoked by RCS-RF. All these substances except dexamethasone also prevented the conversion of exogenous arachidonic acid into TXA₂, confirming that they act by a direct block of cyclo-oxygenase⁴. Dexamethasone, however, blocked the release of TXA₂ by RCS-RF without affecting the conversion of exogenous arachidonic acid to TXA₂, implying a different site of action. We therefore examined the effects of other steroids in this system. Table 2 shows that all the steroids tested blocked the releasing activity of RCS-RF. Furthermore, their relative activity in this test closely paralleled their reported anti-inflammatory activity. The steroids had variable but generally only small

Mechanism of action and site of steroid blockade

Infusion of arachidonic acid into perfused guinea pig lungs leads to an immediate synthesis of TXA₂ suggesting that substrate availability is a rate-limiting step in this system. RCS-RF may, therefore, act by liberating arachidonate from some intracellular pool, and steroids could be preventing this effect. To test this possibility, we perfused isolated lungs with Krebs' solution containing 5,8,11,14-eicosatetraynoic acid (TYA)—an acetylenic analogue of arachidonate—at 10 µg ml⁻¹. This prevented metabolism of any

Table 3 Release of arachidonate from lungs by RCS-RF

Experiment	Steroid	Dose (nmol min ⁻¹)	Sample collection period (min)	RCS-RF injected	Arachidonic acid efflux from lung (µg)			
					Basal	RCS-RF alone	RCS-RF + steroid	RCS-RF alone
1	None	—	7	5 U (Crude)	0.15	0.22	—	—
2	None	—	7	5 U (Partially purified)	0.18	0.24	—	—
3	None	—	7	5 U (Partially purified)	0.26	0.31	—	—
4	Betamethasone	178.3	5	8 U (Crude)	1.13	5.99	0.78	—
5	Dexamethasone	178.3	7	8 U (Crude)	0.38	1.63	0.43	1.24
6	Fludrocortisone	727.7	7	4.5 U (Purified)	0.78	1.16	0.65	0.78
7	Hydrocortisone	5,517.2	7	4.5 U (Partially purified)	—	1.56	0.52	1.92

Lung effluent was collected for 5–7 min following an injection of RCS-RF either in the presence or absence of blocking doses of steroids. The pH of the effluent was adjusted to 3.0 with 1 N HCl and the arachidonate extracted with ether, a small amount of 1-¹⁴C-arachidonate was added as a tracer. After concentration of the sample *in vacuo* the fatty acids were methylated with diazomethane and separated on thin-layer chromatography silica gel plates impregnated with 10% AgNO₃ (Uniplates, Anachem) by development in *n*-hexane, diethyl ether, acetic acid (50:50:1 v/v). The methyl arachidonate zone was located with a radiochromatogram scanner and eluted with ether, and the arachidonate content was estimated with a Pye Unicam series 104 gas chromatograph, using a 5-foot column packed with 10% DEGS on Gas-Chrom Q. With an oven temperature of 195 °C methyl arachidonate was easily detected by the flame ionisation detector, having a retention time of about 8 min. The labelled arachidonic acid was used to correct for losses during sample manipulation. Large doses (5–10 U) of RCS-RF were used to ensure that easily detectable amounts could be liberated, and correspondingly higher doses of steroids were used to compensate for this.

effects on the conversion of arachidonic acid to TXA₂, which were unrelated to their anti-RCS-RF activity. The release of TXA₂ by arachidonate, for example, was unaffected by dexamethasone, potentiated by triamcinolone and depressed by fludrocortisone. These effects could be related to the vehicle in which the steroid was dissolved; for ethanol (used to dissolve some of the steroids) in concentrations greater than 10 µl ml⁻¹ depressed the release of TXA₂ by arachidonate.

There were two other notable features concerning the block of TXA₂ release by anti-inflammatory steroids; first, a maximal effect was obtained only after infusing each steroid for 10–20 min. Second, the inhibition produced by steroids was easily overcome by increasing the amount of RCS-RF. Thus, the ID₅₀ of a steroid will depend on the concentration of the agonist.

released arachidonate by the lung, TYA being an inhibitor of both the cyclo-oxygenase and lipoxygenase pathways of arachidonate metabolism^{9,10}. Table 3 shows that both "partially" purified and "crude" RCS-RF liberated arachidonate from lungs and that this release was blocked when any of the steroids was present. Thirty minutes after stopping the steroid infusion, arachidonate release by RCS-RF returned, indicating that the effects of the steroids in this system were relatively short lived and easily reversed.

The actual amounts of arachidonate released from lungs varied greatly between experiments (see Table 3, experiments 4–7) but were relatively consistent in single lungs (experiments 1–3). Although the samples were collected for 5–7 min it should be realised that the evoked release of arachidonate usually takes place within 1 min giving an effect similar to a bolus injection of the substrate. It is

interesting to note that there is a continuous basal release of arachidonate from the isolated lungs. This was generally found to increase with time (experiments 1-3, Table 3). In one experiment (No. 4) at least, betamethasone actually lowered the stimulated release below basal levels. This observation correlates with an effect frequently observed during steroid infusion—a decline in tone of the bioassay organs, suggesting a decreased basal TXA_2 output.

What is the source of the arachidonic acid liberated by RCS-RF? Although amounts of free arachidonic acid in many tissues are small (see ref. 11) considerable quantities are present in ester form in the neutral lipid or phosphatide fractions. Several observations (see ref. 11) indicate that phospholipids are the chief source of arachidonate in cells, so that the phospholipase enzyme system may be stimulated in some way by RCS-RF. Some evidence for this possibility has already been obtained in this laboratory (G. J. Blackwell, R.J.F. and F.P.N., unpublished). Specifically labelled ($2'-(1-^{14}\text{C})$ -oleoyl) lecithin is partially ($\sim 1-3\%$) hydrolysed when perfused through the isolated lung—presumably by a phospholipase A_2 . The rate of hydrolysis is doubled when concomitant injections of 5 URCS-RF were given, and decreased when infusions of dexamethasone or betamethasone ($178.8 \text{ nmol min}^{-1}$) were made. Phospholipase A_2 activity in guinea pig lung homogenates, however, was not affected by the anti-inflammatory steroids, suggesting that these drugs only have a blocking action in intact cells.

RCS-RF and anaphylaxis

We do not know whether RCS-RF formation and release contributes to anaphylaxis in guinea pigs. It does not seem to have any intrinsic smooth muscle activity in the doses we used here. Our finding that it cannot be recovered from the lungs of unsensitised animals or from unchallenged sensitised lungs would suggest that it is not stored in the lung, but is elaborated in response to the immunological stimulus. Thus, if it does have a role in anaphylaxis it could be among those substances classified as “primary mediators” by Austen and Orange¹². If it is a peptide, RCS-RF cannot be the SRS-A of Orange *et al.*⁷, a conclusion reached earlier by Piper and Vane¹ on the basis of biological evidence. Although there seem to be similarities between SRS-A and RCS-RF, any formal relationship between the two compounds (as suggested by the arylsulphatase inactivation experiment) awaits further clarification.

Of great interest is the finding that anti-inflammatory steroids block the RCS-RF induced release of thromboxane. Even though they do not inhibit the cyclo-oxygenase¹³ there has been increasing evidence that one steroid (hydrocortisone) interferes in some way with prostaglandin biosynthesis in several other systems¹⁴⁻¹⁷. The mechanism proposed by Lewis and Piper¹⁴ to account for hydrocortisone action, that of an inhibition of a cellular transport mechanism, does not operate here since arachidonate consistently reversed the steroid block. Instead, our results point to a mechanism of action much earlier in the biosynthetic pathway—the inhibition of substrate liberation from intracellular stores. A similar hypothesis has already been advanced by Gryglewski and his colleagues¹⁵. This store is probably the phosphatide fraction of the cell, and the enzyme responsible for liberation of the acid, the cell membrane phospholipase A_2 . Bradykinin is another peptide which releases RCS from the lung¹⁸ and, like RCS-RF, its activity is potentiated by BPP-5A. We find, however, that only RCS-RF activity is steroid sensitive, implying that it activates a very selective enzymatic step.

The order of potency of the steroids in inhibiting RCS-RF activity is striking similar to the order of anti-inflammatory potency, and the possibility that inhibition of substrate release is a general method by which anti-inflammatory steroids can interfere with the synthesis of prostaglandin (or other arachidonate metabolites) must be considered.

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letters to nature

Cosmic rays near the galactic centre

THE galactic centre (GC) is characterised by objects of high activity: there are powerful sources of infrared radiation, radio waves and X rays, and evidence for a small region ($\sim 1 \text{ pc}$) of extremely high luminosity ($\sim 10^6 L_\odot$). The presence of a ring of gas (very largely molecular hydrogen)¹ at a distance of $\sim 300 \text{ pc}$ from the GC also indicates violent activity. It is reasonable to assume that there is also an increased cosmic-ray intensity at least for energies $\lesssim 10 \text{ GeV}$. The presence of protons and electrons having such energies together with high gas densities and high starlight intensities provides the ingredients for significant γ -ray fluxes. Here we examine the implications of recent measurements of the γ -ray flux from the region of the GC, with particular reference to the 300-pc ring.

The most comprehensive γ -ray data so far obtained are those from the SAS-II satellite (see ref. 2). The earlier data did not give strong evidence for a peak at $l^{II} \sim 0^\circ$ but a very recent reanalysis by Fichtel *et al.*³ shows a rather impressive peak (see Fig. 1) which offers the possibility of deriving the cosmic-ray intensity in the GC region. It should be remarked, however, that the only other precise cosmic γ -ray experiment, that on the COS B satellite⁴, does not show a peak near $l^{II} = 0^\circ$. The analysis so far is described as “preliminary”, however, and we are of the view that the peak is still very probably genuine.

Although the SAS-II peak looks rather broad ($\text{FWHM} \approx 6^\circ$) most of this is instrumental and it seems that the bulk of the radiation is coming from within about $\pm 2^\circ$ of the GC. The distribution thus seems to be both narrower and of higher

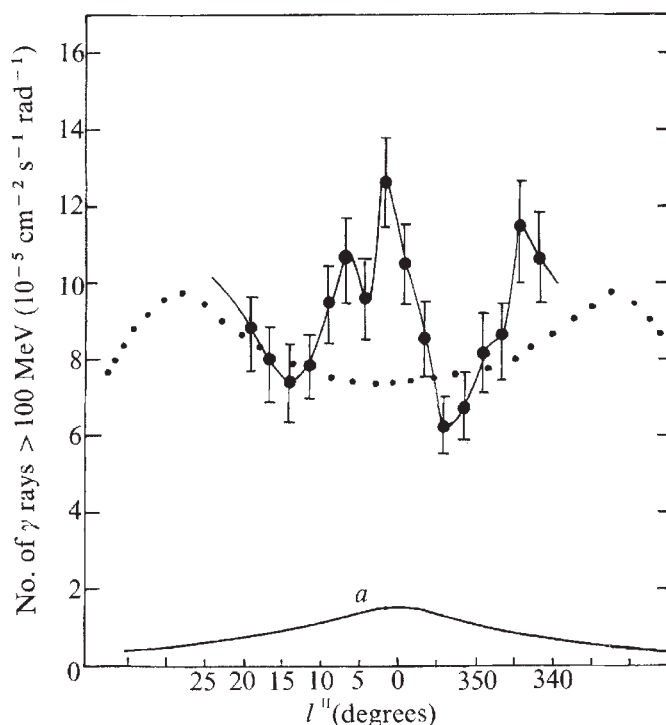


Fig. 1 The flux of γ rays above 100 MeV for longitudes close to the GC, from Fichtel *et al.*³. ••• Estimate of the contribution for $R > 350$ pc, where we assume that the cosmic-ray intensity is proportional to the mean surface gas density. *a*, Contribution from electrons by way of the inverse Compton effect, assuming a constant electron flux throughout the Galaxy⁶.

intensity than estimated for inverse Compton interactions by Dodds *et al.*⁵ (see Fig. 1) for the case of an electron intensity near the GC similar to that locally (and with a full width for the electron distribution of 500 pc). There is thus evidence for an increase in the cosmic-ray intensity near the GC.

The original SAS-II data have been analysed by many authors and it has been shown that there is almost certainly a gradient of 1–10-GeV cosmic-ray protons in the Galaxy. Our view is that the firmest evidence comes from the analysis of measurements towards the galactic anti-centre⁶ but the most reasonable interpretation of γ rays from the inner Galaxy is that there is a gradient there too, with the cosmic-ray intensity roughly following the average surface gas density⁷ and the surface density of supernova remnants and pulsars^{8,9} (at least for $R \gtrsim 4$ kpc). Such a correlation would be expected if the cosmic-ray lifetime is roughly constant for $R \gtrsim 4$ kpc, in which case the intensity will be proportional to the generation rate.

Near the GC we might expect the generation rate to be again roughly proportional to the gas density, and in what follows we examine whether this is the case.

The SAS-II data of Fig. 1 indicate a flux of γ rays from within a few degrees of the GC of $\sim 6.7 \times 10^{-6} \text{ cm}^{-2} \text{ s}^{-1}$. This value has been derived using as a datum for non-centre flux the distribution shown dotted in Fig. 1; in the calculation the contribution from the rest of the Galaxy followed from the assumption that the cosmic-ray intensity is proportional to mean surface gas density.

Locally, the escape times for electrons and protons are roughly equal and therefore the e-p ratio is expected to be the same as at generation. In the GC region the lifetime against energy loss is about the same for electrons and protons (see later) and so we expect their intensities to be enhanced by the same factor (F) over their local values. The procedure is to calculate F so that the measured flux of γ rays at the Earth is reproduced.

The first contribution arises from the decay of π^0 s produced by protons and heavier cosmic-ray nuclei interacting with the

interstellar matter at the GC. For the mass of gas and its distribution we use the measurements of Scoville and Solomon¹⁰ which indicate that there is about $5 \times 10^7 M_\odot$ of molecular hydrogen distributed within a radius of ~ 350 pc and a volume of $\approx 10^7 \text{ pc}^3$. Assuming that the cosmic-ray spectrum has the same spectral shape as that locally, the expected flux of γ rays above 100 MeV at the Earth is $\sim 6.8 \times 10^{-7} F \text{ cm}^{-2} \text{ s}^{-1}$. Although there will be a contribution from inverse Compton interactions of electrons in the starlight field, in the present model this will be outweighed by the yield from bremsstrahlung interactions. We assume that the lifetime for escape of the cosmic-ray nuclei from the ring is significantly greater than 10^5 yr, the lifetime against interaction. The protons will then be in equilibrium with the secondary electrons from $\pi \rightarrow \mu \rightarrow e$. The electrons, too, are assumed to be absorbed before escaping from the ring, bremsstrahlung predominating (the mean lifetime is $\sim 2 \times 10^5$ yr at 1 GeV). Ramaty¹¹ has given the generation spectrum for electron secondaries and from this the secondary electron intensity has been derived. As an example, the derived intensity at 1 GeV is $\sim 12\%$ of that of primary protons in the ring. To this must be added $\sim 3\%$ for primary electrons giving a total electron intensity at 1 GeV in the ring of 15% of the proton intensity.

At electron energies of ~ 100 MeV ionisation losses predominate over bremsstrahlung and the γ rays at 100 MeV are mainly from π^0 decay. This implies $F \approx 10$.

A value for F (to be denoted F_e) can also be calculated from non-thermal radio observations of the GC if we assume that there is a coupling between the cosmic-ray energy density and that in the magnetic field. Measurements at 408 MHz (ref. 12) suggest a brightness temperature of 100 K from the 300-pc molecular ring ($\sim 4 \times 10^{-4} \text{ sr}$ region, of which we estimate $\sim 25\%$ to be thermal¹³). Using a lifetime against bremsstrahlung of 2×10^5 yr, the equilibrium electron intensity has been calculated. Equating the calculated synchrotron flux to observations gives $F_e \approx 12$. This is rather larger than the value of F calculated from the γ -ray flux but it is not inconsistent considering uncertainties in the electron lifetime and the likely underestimate of the percentage of thermal radiation.

The value $F = 10$ derived from the γ -ray analysis can be used to derive the relative generation rates in the ring, where the lifetime is $\sim 10^5$ yr, and locally, where we assume that the lifetime by escape is $\sim 2.5 \times 10^6$ yr. The ratio ring-to-local value is ~ 250 ; this can be compared with the ratio of gas densities, $\sim 200:1$. The nearness of the results adds some weight to our original contention.

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The 35-d X-ray profile of Her X-1

FOLLOWING the report from Ariel V (ref. 1) of substantial X-ray emission from Her X-1 midway through the extended low-state portion of its 35-d cycle, Jones and Forman² have reported a similar feature during another cycle, measured from Uhuru. They further suggest that this feature should occur regularly, consistent with models for the mass accretion disk which are inclined with respect to the binary orbital plane^{3,4}. We report here the results of the analysis of > 500 d of Ariel V all-sky monitor (ASM) data, which support the view that the feature occurs regularly in the 35-d cycle.

The Ariel V ASM is barely sensitive to Her X-1 under the most favourable of viewing constraints (see ref. 5 for a complete description of the apparatus). Its sensitivity for accumulation times ≥ 0.5 d is no better than ~ 0.1 the intensity of the Crab Nebula (approximately the maximum intensity of Her X-1), so that considerations, such as occultation by the Earth, make the average sensitivity of the experiment even poorer. During the total duration of observation reported here (October 16, 1974 through April 16, 1976), a total of 803 data accumulations of ≤ 0.5 d, for which Her X-1 was not either outside or at the very edge of the experiment field-of-view, have been obtained. Only 24 of these allow intensity definition at the 2σ level (corresponding to $\sim 3\sigma$ detection of a positive signal above background). Of these 24 measurements, all but one were within the 'on-time' of the 35-d cycle, and a full 1/3 were obtained from a single cycle during which the viewing constraints were near-optimum. It is important to note that the same maximum source intensity produces, for average viewing conditions, a $< 1\sigma$ signal.

We can, however, take advantage of the fact that we can differentiate between null statistical results under optimum viewing conditions (corresponding to a knowledge that the source was not at maximum intensity), and those at more unfavourable conditions (for which no such knowledge can be gleaned). All the data were, therefore, normalised to the same viewing constraints, with individual negative measurements of

the intensity relative to background initially defined to be zero. These 803 points, excluding measurements centred within 7 per cent of the 1.7-d Her X-1 eclipse minimum, were then folded at various trial periods near 35 d. In Fig. 1, where we have plotted the total variance for a 15-bin light curve compared to the average value (essentially equivalent to a χ^2 test against the hypothesis of a constant source intensity), the 35-d modulation of the normalised record is apparent. Figure 1 alone prescribes a period of 34.7 ± 0.3 d (where the width of the variance peak is consistent with the overall sample duration), in agreement with the 34.88 ± 0.12 d of ref. 6. A comparison of the shape and phase of the on-state portion of the light

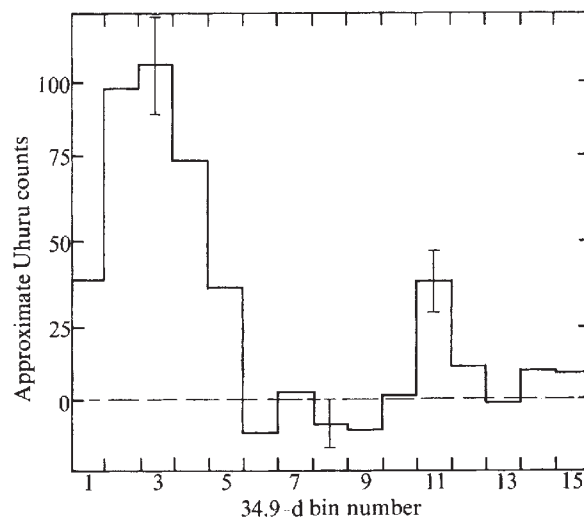
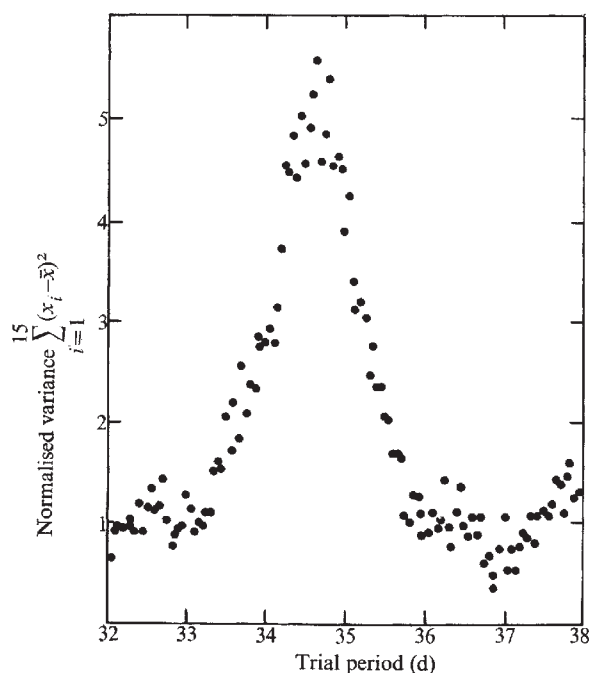


Fig. 2 34.9-d light curve of Her X-1 in 15 bins. The ordinate is normalised to approximate Uhuru counts for comparison with other measurements. The epoch of the start of bin 1 is JD 2,442,442.0.

Fig. 1 Variance for 15-bin light curves as a function of trial period. The ordinate is normalised to the mean for trial periods 32.0–33.0 d and 36.0–38.0 d.



curves obtained here with those of ref. 6 clearly favours a value of 34.9 ± 0.1 d.

The 34.9-d light curve is exhibited in Fig. 2, where the negative intensity measurements are now properly included in the folding procedure (allowing both consistent error estimates and a zero baseline intensity to be obtained). In addition to the characteristic reproduction of the on-state portion of the cycle (bins 1–5), bin 11 contains a $> 3\sigma$ excess relative to the surrounding off-state intensity. This feature does not arise primarily from the single off-state measurement of high statistical significance, as that datum contributes to bin 12. Jones and Forman have reported that the analogous feature they observed in Uhuru data near January 1, 1972 represented $\sim 30\%$ of the maximum intensity, with a duration of $\sim 15\%$ of the 35-d cycle (between $\phi = 0.45$ and $\phi = 0.6$ relative to $\phi = 0$ at maximum). The present data agree with the magnitude of the effect, but differ somewhat in phase and duration. In the Ariel V data, the feature is centred at $\phi = 0.55 \pm 0.05$ relative to maximum, with an apparent duration of $\leq 10\%$ (at an intensity $\geq 1/4$ of maximum). The agreement in magnitude of the present feature with that obtained from Uhuru, coupled with the fact that it is never statistically detectable in an individual measurement, strongly implies that it occurs regularly in every cycle at $\sim 1/3$ of maximum.

Jones and Forman have attempted to refine model disk geometry parameters on the basis of the Uhuru data, but the present measurements are obviously too crude to revise sensibly their estimates. Clearly, a high-sensitivity measurement of this portion of the light curve is required, and our results would suggest that this experiment is possible during any Her X-1 cycle.

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Microwave spectral lines from interstellar dust

SPECTRAL features at 3.07, 10, 20 and 33 μm have proved invaluable in the chemical characterisation of interstellar dust^{1,2}. As spectral observations are extended into the far infrared, it is likely that other features attributable to the absorption and emission of radiation by dust will be observed. To date there has been no discussion in the literature on the possibility of discrete spectral features attributable to dust at even longer wavelengths. The purpose of this note is to suggest that relatively sharp spectral lines may arise from neutral or ionised atomic species in interstellar dust at mm and cm wavelengths. The detection of these spectral features would provide definitive information on the chemical composition of interstellar dust.

The 21-cm line of atomic H arises from the $F=0 \longleftrightarrow F=1$ hyperfine transition of ground state H atoms. H atoms can be found in many different types of solid or on solid surfaces (Table 1) when these solids are subjected to nuclear irradiation. The $F=0 \rightarrow F=1$ transition for H atoms in these systems occurs at the frequencies shown in Table 1. The list has been chosen as representative of solids of astrophysical interest, but it is by no means complete. It serves only to show that H atoms can be stabilised in many types of solid astrophysical interest. H atoms in these environments will absorb or emit radiation at frequencies within $\pm \sim 30$ MHz of 1,420 MHz. The width of the hyperfine line as measured in ESR experiments is typically 0.3–3 MHz. This width arises primarily through spin-lattice coupling³ for temperatures $\lesssim 50$ K and would be smaller in an astrophysical environment where coupling to lattice acoustic phonons will be diminished, because of the small size of interstellar grains. This effect, which has been seen⁴ in laboratory ESR spectra of powders, arises because spin-lattice relaxation at low temperatures involves the creation

of acoustic phonons whose energies are close to that of the hyperfine transition. In small particles a cutoff of the acoustic phonon spectrum occurs at $v_c \approx v_s/2a$ where v_s is the speed of sound in the solid⁵ ($\approx 10^5$ cm s⁻¹) and a is the particle radius. The value of $v_c \approx 5 \times 10^9$ Hz, for $a = 10^{-5}$ cm; and $\approx 5 \times 10^{11}$ Hz for $a = 10^{-7}$ cm. Both these frequencies are $> 1,420$ MHz, and thus single-phonon spin-lattice relaxation would not be important for H atoms in interstellar grains. Broadening due to spin-spin interactions, which is proportional to spin concentration³, could be important in larger particles but would not be expected to be significant in small particles containing only a few H atoms. We conclude that $\Delta\nu \approx 0.3$ –3 MHz is likely to be an upper limit to the width of the 21-cm line for H atoms trapped in interstellar grains.

Table 2 Representative data on zero field transitions of Fe³⁺ and Cr³⁺ ions in various crystals

System	ν (GHz)	$\Delta\nu$ (MHz)	Ref.
Fe ³⁺ —SBQ*	7.1132 $\pm$ 0.0002 8.8125 $\pm$ 0.0002	10–20	15
Fe ³⁺ —NA†	24.1575 $\pm$ 0.0005 35.365 $\pm$ 0.002	10–20	15
Fe ³⁺ —Al ₂ O ₃	12.07 19.13		16
Fe ³⁺ —TiO ₂	43.3 81.3		17
Fe ³⁺ —MgO	1.844		Calculated
Fe ³⁺ —CaO	0.5846		Calculated
Fe ³⁺ —CaCO ₃	6.550 $\pm$ 0.4 11.03 $\pm$ 0.18		18
Cr ³⁺ —Al ₂ O ₃	11.5		19
Cr ³⁺ —TiO ₂	43.3		20
Cr ³⁺ —MgAl ₂ O ₄	29.7		21

*SBQ, Synthetic brown quartz.

†NA, Natural amethyst

H atoms could be formed in interstellar grains through the action of cosmic rays or photons from the interstellar ultraviolet radiation field. Alternatively, H atoms from the ambient gas may collide with and stick to grains. To estimate the column density required to yield measurable absorption one can use the relationship between $n_H L$, the number of H atoms in grains per cm², and the optical depth τ_v given by Kerr⁶.

$$n_H L = 3.88 \times 10^{14} T_s \tau_v \Delta\nu$$

where T_s is the excitation temperature, and $\Delta\nu$ is the linewidth in Hz. Taking $T_s = 10$ K as typical of the temperature of an interstellar grain, $\tau_v = 10^{-2}$ and $\Delta\nu = 10^5$ Hz, one has $n_H L = 3.9 \times 10^{18}$ cm⁻². Then, $n_g L$, the column density of grains = $3.9 \times 10^{18}/n$ where n is the number of H atoms per grain. For a 1% concentration of H atoms in grains one obtains $n_H L \approx 10^{24}$ cm⁻² independent of the choice of a when the usual mass ratio of gas to dust is used and the dust is assumed to consist of a material with density ≈ 2 g cm⁻³ made up of the heavier elements. Though column densities as great as this are not found in typical interstellar clouds, they may be found in protostar clouds or regions of strong infrared absorption or emission⁷.

Zero-field splitting³ of the ground state of many transition metal ions in solids occurs when the crystal field partially removes the M_J degeneracy of states with $J > 1/2$. As an example, the ground state of Fe³⁺ is $^6S_{5/2}$ in the free ion. In a crystal with other than cubic symmetry, this state splits into 3 doubly degenerate levels with $m_J = \pm 1/2, \pm 3/2$ and $\pm 5/2$. Magnetic dipole transitions with $\Delta m_J = 0, \pm 1$ are permitted between these levels. Table 2 shows that these transitions occur in the frequency range between 6 and 80 GHz. In a crystal field with cubic symmetry (MgO, CaO) the ground state of Fe³⁺ splits into only 2 levels, and in that case a single spectral line is ob-

Table 1 Frequency of H atom $F=0 \rightarrow F=1$ hyperfine transition in various solids

System	ν (MHz)	$\Delta\nu$ (MHz)	T (K)	Ref.
H in H ₂	1,417.11	1	4.2	7
H in silica	1,409.1	1.4–2.8	78	8
H in crystal quartz	1,453.1 $\pm$ 0.1	$\approx$ 0.3	78	8
H on silica surface	1,409.3	< 3	77	9
H on silica gel surface	1,411.5		77	10
	1,417	≈ 3	77	
H in H ₂ O	1,400	$\approx$ 20	4	11
H on SiO ₂ : Al ₂ O ₃ surface	1,416	≈ 3		12
H on NH ₄ Y and HY zeolites	1,400	≈ 9		13
	1,406	≈ 3.5		
H in CH ₄	1,412	$\approx$ 45	4.2	14

served. Taking a spectral line at $\nu = 100$ GHz with $\Delta\nu = 10$ MHz as typical of these transitions one obtains

$$n_{Fe}L \simeq 2 \times 10^9 T_s \tau_\nu \Delta\nu$$

where

$$T_s = 10 \text{ K}, \tau_\nu = 10^{-2}, \text{ and } n_g L \simeq 2 \times 10^{15}/n$$

where n is the number of Fe^{3+} ions per grain. The corresponding hydrogen column density is $N_H L = 10^{21} \text{ cm}^{-2}$ when one assumes a Fe^{3+} concentration in dust of 1%. Our conclusion is that transitions between the zero-field levels of Fe^{3+} or other transition metal ions in interstellar dust may be observable in absorption in typical interstellar dust clouds.

Though we have emphasised the possibility of detecting absorption features attributable to dust in the mm and cm regions it may be more practical to search for emission features. Grains should be relatively transparent in this region, because of the absence of optical phonon modes at such low frequencies. This implies that most of the energy radiated by grains at mm and cm wavelengths will be radiated within these spectral lines. In view of the optical pumping of interstellar grains that occurs through absorption of short-wavelength photons from the interstellar radiation field, non-thermal emission at the frequencies of these discrete features is a distinct possibility. Microwave lines at 81.541 GHz and 43.122 GHz, attributed to emission from rotational levels of the $SiO \nu = 1$ state²², are both within 0.2 GHz of the zero-field lines from Fe^{3+} - TiO_2 (Table 2).

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Force-free magnetic fields in the fluid interiors of neutron stars

I show here that, in equilibrium, the magnetic field in the fluid interior of a neutron star satisfies the condition $\mathbf{B} \times \nabla \times \mathbf{B} = \mathbf{0}$, which is the usual condition for a field to be force free¹. The significance of this result is that it places a great theoretical restriction in the possible structure of such fields. Restrictions such as this are important because it is still very difficult to deduce anything from observation about the magnetic fields inside neutron stars.

In the region of the star considered here, the density lies between approximately $2 \times 10^{14} \text{ g cm}^{-3}$ and a few times this

value. The material in that region is a fluid composed of neutrons, protons and electrons^{2,3}. It is a simple matter to extend the proof to include the effects of muons and hyperons which may appear at higher densities². Although calculations indicate that the neutrons are superfluid and the protons are superconducting at the temperatures expected inside neutron stars^{2,3}, the proof of the force-free nature of the field does not depend on these assumptions. Newtonian gravitational theory is used here; the general relativistic proof is essentially similar. The proof may also be extended to rotating neutron stars by including centrifugal terms in the chemical potentials.

The crucial part of the argument is that the dynamical β -equilibrium between the neutrons, protons and electrons requires that

$$\mu_e + \mu_p = \mu_n \quad (1)$$

where the μ s are chemical potentials. These potentials include rest mass energies, Fermi energies and gravitational potential energies per particle. The potentials also depend to a very slight degree on the magnetic field strength⁴. The equilibrium of the neutrons implies that their chemical potential is constant throughout the region

$$0 = -\nabla \mu_n \quad (2)$$

This equation expresses the hydrostatic equilibrium of the neutrons.

But $-\nabla \mu_e$ and $-\nabla \mu_p$ do not vanish in the presence of a magnetic field, unlike $-\nabla \mu_n$, because both the electrons and protons experience Lorentz forces. If the electron and proton analogues to equation (2) are added together, the result is an equation expressing the magnetohydrostatic equilibrium of the electron-proton plasma

$$0 = -\nabla(\mu_e + \mu_p) + \frac{1}{cn_p} \mathbf{J} \times \mathbf{B} \quad (3)$$

In deriving the equation, which holds in the centre-of-mass frame of the plasma, charge neutrality ($n_e = n_p$) has been used. It follows directly from equations (1), (2) and (3) that

$$\mathbf{J} \times \mathbf{B} = \mathbf{0} \quad (4)$$

where the conduction current density is denoted $\mathbf{J}$. No distinction is made here between the magnetic field $\mathbf{H}$ and the magnetic induction $\mathbf{B}$ because the magnetic permeability is effectively unity. At the densities considered here, the spacing between Landau levels is very much smaller than the Fermi energies (V. Canuto, unpublished). In addition, for a wide range of reasonable temperatures and field strengths, the spacing is smaller than, or comparable to, kT . As a result of these conditions the de Haas-van Alphen oscillations in the magnetic permeability can be neglected.

Because of the previously noted slight dependence of the chemical potentials on the magnetic field strength, there is a small force on the plasma attributable to the magnetic field. But for the reasons mentioned with regard to the magnetic permeability, this force is relatively insignificant (ref. 4, and I.E., unpublished).

It follows from equation (4) that $\mathbf{B}$ satisfies

$$\mathbf{B} \times \nabla \times \mathbf{B} = \mathbf{0} \quad (5)$$

which is known as the 'force-free' condition¹.

It should be noted that this condition on $\mathbf{B}$ is true in spite of a small force on the plasma, resulting from the field. The reason the magnetic fields in many other astrophysical plasmas are believed to be force free or nearly so is the smallness of the plasma pressure compared to the magnetic pressure¹; the

reason for the force-free nature of the field in the neutron star fluid interior is entirely different. The essential point here is that in β -equilibrium, the electrons and protons 'communicate' with each other through the neutrons, causing $-\nabla(\mu_e + \mu_p)$ to vanish, in turn causing $\mathbf{J} \times \mathbf{B}$ to vanish. Furthermore, since the standard stability analyses of force-free fields do not take rotation into account¹, they are not applicable to many neutron stars, in which rotation may profoundly affect the interior plasma dynamics (refs 5 and 6, and I.E., unpublished).

As one simple example of the theoretical use to which equation (5) can be put, note that it can eliminate from consideration any non-singular, purely toroidal field, such as that used by Vandakurov⁷.

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A rare event in the stratosphere

PERTINENT to the question of the existence of haze and water vapour in the stratosphere^{1,2} was the detection of a nacreous cloud at 25 km over New Mexico on June 20, 1974. This occurred just before the volcanic dust incursion from Fuego in November, 1974 (ref. 3), during a high altitude balloon experiment in which a large polar nephelometer was used to measure the optical angular-scattering characteristics of atmospheric aerosols⁴.

The balloon launch was made at 0748 MST from Holloman AFB, New Mexico (32°N, 107°W) into a very clear sky. The nephelometer reached an altitude of 26.6 km and

payload ground-impact was 7 h later near Silver City, New Mexico, 210 km W of the launch site. Easterly winds of ~ 20 knots carried the experimental package on a trajectory perpendicular to the San Andres mountain range. The minimum ambient of -75°C was reached at 16.8 km, and the balloon was still ascending when the very strong signal was encountered on the lee side of the mountains. The intense signals continued to the apogee of the flight at 26.6 km without diminution, thereby implying a greater vertical extent for the increased aerosol particle concentrations. The balloon was allowed to float at this maximum altitude for 15 min, evidently immersed in the cloud. Moreover, the signals continued for a period of 50 min, during which time the balloon had travelled nearly 50 km with respect to the ground, thus making the latter distance the minimum horizontal extent of the cloud. On the descent of the balloon, the scattering ceased as dramatically as it had developed on ascent.

Figure 1 shows a typical altitude profile of the volume scattering function (the absolute intensity) measured at a scattering angle of 15° and spectral wavelength of $0.475 \mu\text{m}$. Four other photometers, at angles of 30° , 50° , 100° and 150° gave a similar intensity enhancement, although proportionately reduced by the characteristic forward trend of scattering from large particles. The high intensity at ground level (1.29 km above sea level) is caused by exhaust smoke from the balloon launch crane, while that from 3-7 km results from increased amounts of aerosol particles in the convective mixing layer. The stratospheric haze is graphically illustrated by the large variations in intensity as a result of altitudinal inhomogeneity in the particle concentration. The payload also apparently descended through a Cirrus cloud at ~ 10.6 km.

The unusual occurrence of a large haze cloud at 25 km—though it was not evident from the ground—was fortuitous, and is rarely observed in summer at mid-latitudes. Stanford and Davis⁵ have made a review of stratospheric cloud reports for the period 1870-1972, and of some 183 sightings only 3 were reported in the USA. Of these three, two events occurred near the San Andres mountains and the remaining one in northern Arizona. This suggests that the mountains of New Mexico may cause the development of stratospheric clouds; however, their visual observation is

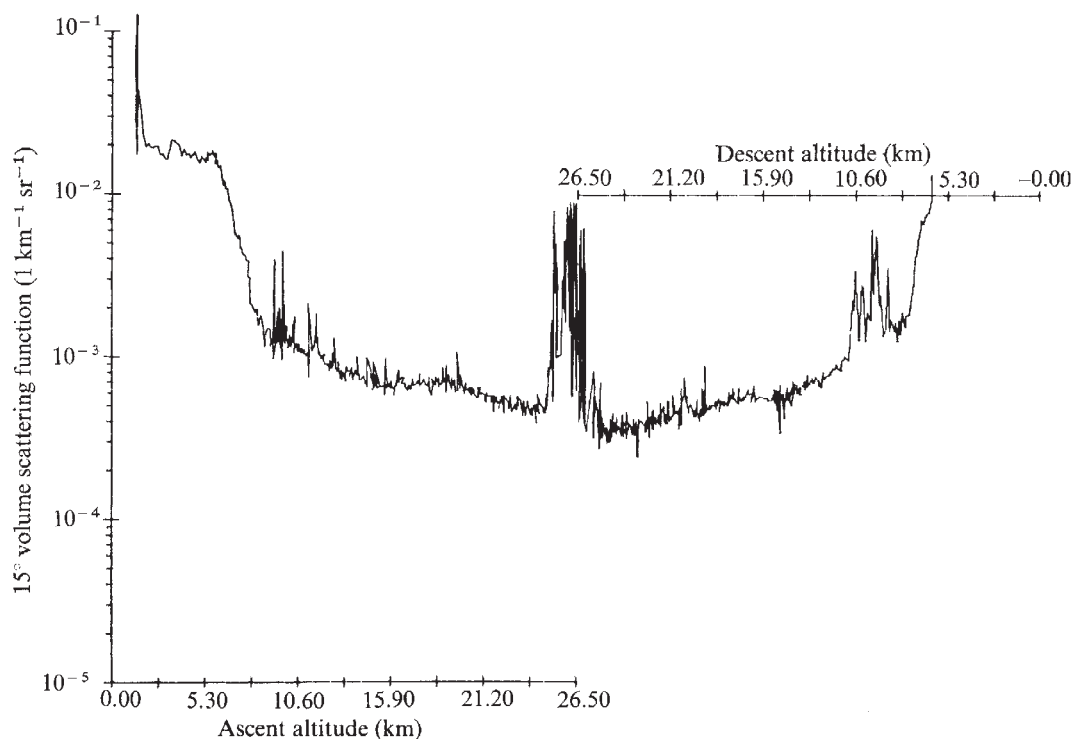


Fig. 1 The 15° volume scattering function against altitude at a wavelength of $0.475 \mu\text{m}$.

difficult because of their high altitude and tenuous structure. Nonetheless, the rarity of sightings may result in part simply from a lack of observation facilities (or an observer) rather than infrequency of occurrence of such events.

Finally, this cloud provides experimental evidence relevant to Scorer's² suggestion, that, even in the absence of volcanic dust, air-mass interchange mechanisms across the tropopause can result in extensive haze formations at high altitudes. Moreover, observations made *in situ* logically provide more reliable information on the stratospheric optical environment, since atmospheric dynamics in the troposphere not only produce sundry effects in the stratosphere but complicate, in particular, remote measurements, since scattering properties along the ground-to-stratosphere light transmission path must be considered.

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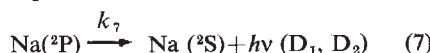
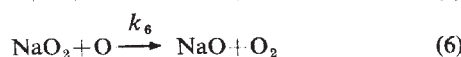
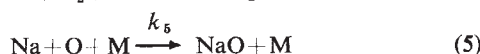
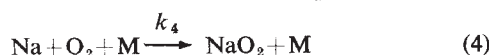
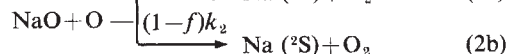
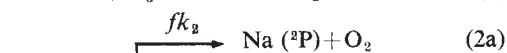
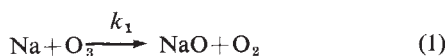
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Gas phase chemical kinetics of sodium in the upper atmosphere

THE existence of a layer of atomic sodium in the upper atmosphere near 90 km has been clearly demonstrated by analyses of daytime and twilight airglow emissions of D-line radiation at 5,890 and 5,896 Å (refs 1 and 2) as well as by direct laser backscatter measurements (see, for example ref. 3). Following Chapman⁴ chemical reactions involving atomic sodium and atmospheric oxygen species have been invoked to explain the chemical dynamics of the sodium layer and the nightglow emission of D-line radiation. In addition, Chapman has also proposed that similar reactions involving newly ablated sodium are responsible for the occurrence of long enduring visible meteor trails⁵. Unfortunately, previous quantitative attempts to model sodium-induced upper atmospheric chemiluminescence have suffered from rather poor estimates of the appropriate reaction rate constants. Here we provide more reasonable estimates of rate constants and show how these new kinetics parameters can dramatically alter calculations of chemiluminescent efficiency.

The sodium-oxygen atmospheric reaction sequence is:



where all species are in their electronic ground states unless otherwise noted. The combination of reactions 1, 2a and 7 in

Table 1 Ionisation data for sodium and oxygen species

Species	IP (eV)	EA (eV)	Reference
Na	5.14		19
NaO	6.5		12
O ₃		1.99	20
O		1.46	21

the presence of O and O₃ in the upper atmosphere form a chemiluminescent chain and may explain both sodium nightglow and meteor trail emissions. Reaction 7 is sufficiently fast ($k_7 = 1/\tau_{\text{rad}} = 6.3 \times 10^{-9} \text{ s}^{-1}$) that all Na (²P) created in reaction 2a radiates before quenching at upper atmospheric densities. Recent observations of the nightglow D-line profiles are consistent with this sodium oxide chemiluminescent cycle^{7,8}.

Several recent attempts to model sodium-oxygen chemistry in the upper atmospheric sodium layer^{1,2,9} and in meteor trail wakes¹⁰ quantitatively have been published. These efforts have been severely hampered by the fact that experimental rate constants are only available for reactions 5 and 7. To overcome this handicap, most authors have adopted experimental

Table 2 Calculated reactive cross sections, rate constants and exothermicities for sodium-oxygen reactions

Reaction	$\sigma_R (\text{cm}^2)$	$k_R (\text{cm}^3 \text{ s}^{-1})$	$\Delta H_R^0 (\text{kcalorie mol}^{-1})$
1	6.5×10^{-15}	3.3×10^{-10}	-34.8 ± 4
2	2.6×10^{-15}	1.6×10^{-10}	$a - 9.2 \pm 4$ $b - 57.7 \pm 4$
3	3.2×10^{-15}	1.4×10^{-10}	$a - 97.2 \pm 7$ $b - 150.2 \pm 7$

rate constants for reactions of either atomic H or N and OH or NO in place of atomic Na and NaO. Values of the order of 10^{-11} – $10^{-13} \text{ cm}^3 \text{ s}^{-1}$ for k_1 and $10^{-11} \text{ cm}^3 \text{ s}^{-1}$ for $k_{(2a+2b)}$ are typical in these studies.

Unfortunately, these rate constant estimates are very likely to be substantially low. Exothermic bimolecular processes, like reaction (1) between low ionisation potential species such as alkali atoms and electrophilic molecules such as O₃ are known to occur by an electron jump mechanism¹¹. Since the measured ionisation potential of NaO is only $6.5 \pm 0.7 \text{ eV}$ (ref. 12), it is quite likely that reactions (2) and (3) proceed by electron jump mechanisms as well.

Relatively accurate reactive cross sections for exothermic electron jump reactions can be calculated by determining the radius at which the Coulombic attraction of the ion pair formed by the electron jump, equals the energy deficit between the ionisation potential of the metal containing reactant, IP, and the electron affinity of the electrophilic reactant, EA

$$e^2/r_x = \text{IP} - \text{EA} \quad (8)$$

The calculated reactive cross section is merely^{11,13}

$$\sigma_R \approx \pi r_x^2 \quad (9)$$

Using the thermochemical parameters shown in Table 1, equations (8) and (9) can be used to estimate overall reactive cross sections for reactions (1), (2) and (3). Rate constants for these reactions can also be estimated by multiplying the reactive cross sections by the most probable relative velocities for reactant pairs at typical upper atmospheric temperatures.

Table 2 shows reactive cross sections and rate constants for an ambient temperature of 185 K calculated by the procedures outlined above. Table 2 also lists the exothermicities of reactions (1), (2) and (3) calculated using the Na–O bond strength of $60.3 \pm 4 \text{ kcalorie mol}^{-1}$ measured by Hildenbrand and Murad¹², the Na–O₂ bond strength of $65 \pm 3 \text{ kcalorie mol}^{-1}$ measured by McEwan and Phillips¹⁴ as well as O–O and O–O₂ bond strengths

of 118.0 and 25.5 kcalorie mol⁻¹, respectively. Furthermore, the Na-O, Na-O₂ and O-O bond energies can be combined to yield an estimate of the NaO-O bond strength of 122.7 ± 7 kcalorie mol⁻¹.

It should be noted that the rate constants for reactions (1) and (2), listed in Table 2 are significantly larger than the analogous rate constants for hydrogen or nitrogen species used in previous modelling efforts^{1,2,9,10}. As an example of the impact these larger rate constants can have on an atmospheric modelling problem, the upper limit of Na D-line emissions from a ~ 30-g meteoroid with a velocity of 40 km s⁻¹ will be calculated below. Following Baggaley¹⁰ such a meteoroid is assumed to deposit a line density of atomic Na of 2 × 10¹⁵ cm⁻¹. Atmospheric abundances and common molecular diffusion coefficients, *D*, are also adopted from Baggaley's work and are shown in Table 3. The values adopted for O and O₂ number densities are reasonably consistent with recently published measurements¹⁵⁻¹⁷.

The reaction scheme used in the calculation of meteor trail D-line luminosity consists of reactions (1), (2), (3a), (4), (5) and (7) listed above. Calculations were performed with the branching fraction, *f*, in reaction 2 set equal to 1 to yield an upper limit to the D-line emission. Reaction (6) is deleted since the bond values listed above indicate that it is close to thermoneutral and is probably slow at mesospheric temperatures. The exclusion of reaction 6 makes NaO₂ a chain-terminating species in night-time conditions. Only the 'a' channel of reaction (3) was considered. Inclusion of (3b) would have hindered NaO₂ formation and yielded a higher photon production term.

The rate constants for reactions (1), (2) and (3) were used as calculated and listed in Table 2. The rate for reaction (4) has been measured by Carabetta and Kaskan¹⁸ in flame studies and was found to equal 8.2 × 10⁻³⁴ cm⁶ s⁻¹ between 1,420 and 1,600 K, independent of temperature. This rate was adopted for both three-body processes (4) and (5). Reaction (7) is essentially instantaneous on the time scale of mesospheric collisions.

The set of equations describing the interaction of meteor deposited Na with the atmospheric oxygen species may be written

$$\frac{d}{dt} [\text{Na}] = D \nabla^2 [\text{Na}] - k_1 [\text{Na}] [\text{O}_3] + k_2 [\text{NaO}] [\text{O}] - k_4 [\text{Na}] [\text{O}_2] [\text{M}] - k_5 [\text{Na}] [\text{O}] [\text{M}]$$

$$\frac{d}{dt} [\text{NaO}] = D \nabla^2 [\text{NaO}] + k_1 [\text{Na}] [\text{O}_3] - k_2 [\text{NaO}] [\text{O}] - k_3 [\text{NaO}] [\text{O}_3] + k_5 [\text{Na}] [\text{O}] [\text{M}]$$

$$\frac{d}{dt} [\text{NaO}_2] = D \nabla^2 [\text{NaO}_2] + k_3 [\text{NaO}] [\text{O}_3] + k_4 [\text{Na}] [\text{O}_2] [\text{M}]$$

$$\frac{d}{dt} [\text{O}_3] = D \nabla^2 [\text{O}_3] - k_1 [\text{Na}] [\text{O}_3] - k_3 [\text{NaO}] [\text{O}_3]$$

$$\frac{d}{dt} [\text{O}] = D \nabla^2 [\text{O}] - k_2 [\text{NaO}] [\text{O}] - k_5 [\text{Na}] [\text{O}] [\text{M}]$$

$$I = f \Sigma k_2 [\text{NaO}] [\text{O}] \text{ photons s}^{-1} \text{ cm}^{-1}$$

where ∇^2 is the Laplacian operator in cylindrical coordinates. Reaction with meteor-produced atomic oxygen is not included in this model since Baggaley's treatment indicated that this

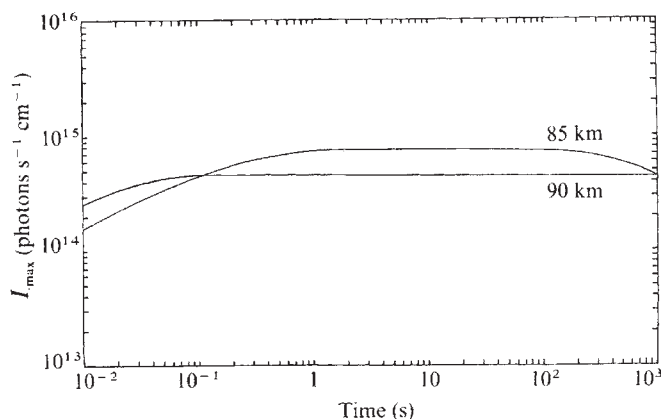


Fig. 1 Maximum D-line photon emission rates at 85 and 90 km.

effect does not persist past the first 10 s¹⁰. An initial meteor train radius of 100 cm was assumed.

Solution of the set of equations listed above was performed using a modified Crank-Nicholson finite difference approach. Solutions of *I_m*, the maximum photoemissions (*f* = 1), for both the 85- and 90-km cases are shown in Fig. 1. The maximum photon emissions found in this study are 4.6 × 10¹⁴ and 7.5 × 10¹⁴ photons s⁻¹ cm⁻¹ at 90 and 85 km, respectively. These compare with peak values of 8 × 10¹⁰ and 3 × 10¹¹ calculated by Baggaley using a *k*₁ of 10⁻¹³ and a *k*₂ of 10⁻¹¹ cm³ s⁻¹. The slow decay of photon intensity after 100 s seen in the 85-km solution is caused by chain-terminating formation of NaO₂ due to the deletion of reaction 6.

The large increases (> 10³) in calculated maximum D-line photon emissions due to the use of more realistic sodium-oxygen reaction rates are clearly important in determining if Chapman's proposed mechanism⁵ can be responsible for long lived visible meteor trails at high altitudes. Although no definitive statement can be made without a firm knowledge of *f*, the branching fraction for excited sodium production in reaction (2), the results shown in Fig. 1 clearly lend more support to Chapman's proposal than Baggaley's recently published calculation¹⁰.

Some feeling for the magnitude of *f* can be obtained by comparing observed nightglow emissions with those predicted by reactions 1-7 using our rate constant estimates. Quantitative precision is difficult because of the wide variations observed in both ambient Na densities and D-line nightglow densities^{2,3,7}; however, a value of *f* between 10⁻¹ and 10⁻² would seem necessary to match observed nightglow intensities.

It should be noted that the rate constants calculated in this work indicate that sodium is an extremely efficient catalytic destroyer of odd oxygen, (O + O₃), at the atmospheric altitudes considered. This efficiency may extend to lower altitudes depending on the magnitude of *k*₄, the photodissociation cross section for NaO₂, and the rate of heterogeneous removal of sodium oxides.

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Table 3 Atmospheric model for meteor trail calculations

Altitude (km)	Diffusion coefficient, <i>D</i> (cm ² s ⁻¹)	Species number densities (cm ⁻³)			
		O ₃	O	O ₂	<i>M</i> (Total)
90	1.0 × 10 ⁵	3 × 10 ⁸	3 × 10 ¹¹	1.3 × 10 ¹³	0.63 × 10 ¹⁴
85	4.2 × 10 ⁴	8 × 10 ⁸	7 × 10 ¹⁰	3.2 × 10 ¹³	1.52 × 10 ¹⁴

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Late Weichselian geomagnetic 'reversal' as a possible example of the reinforcement syndrome

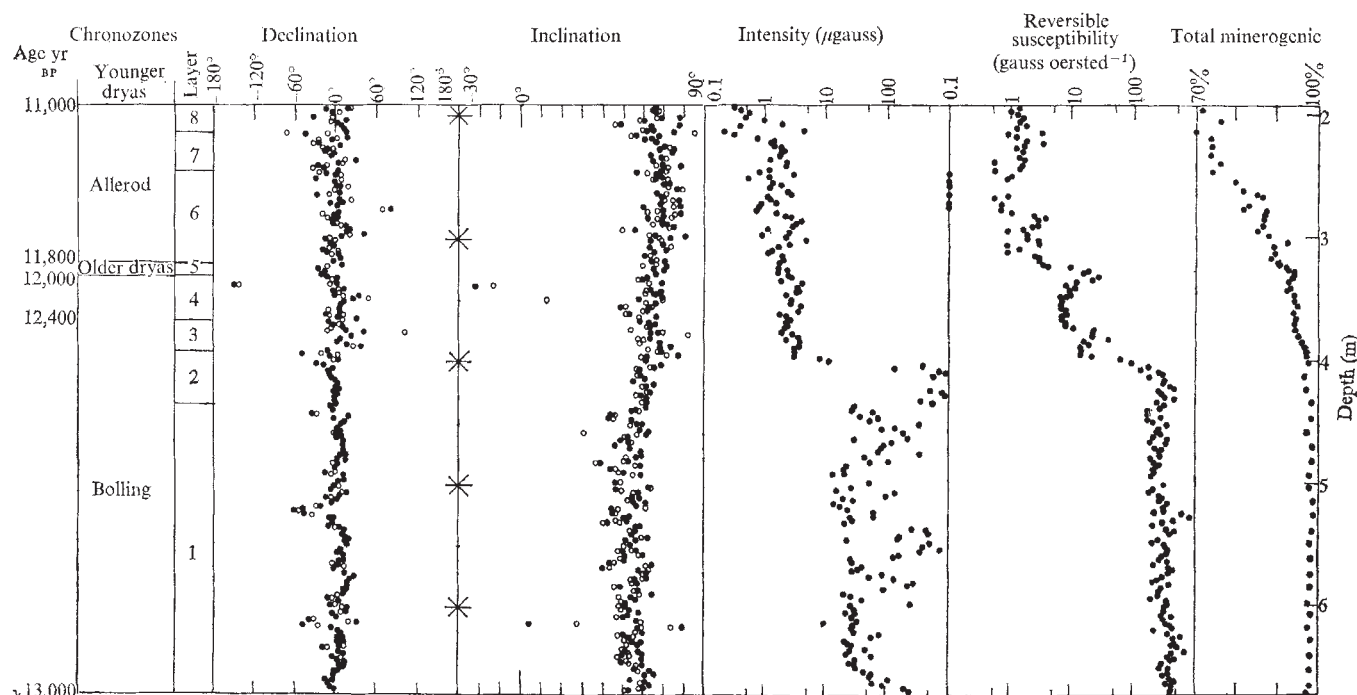
A LATE Weichselian geomagnetic reversal has been reported^{1,2} from Sweden. Mörner and Lanser³ believed the Swedish reversed palaeomagnetic record to be a "real dipole magnetic reversal of greatest significance for global correlations" and to have been established and well dated in Canada, the North Atlantic and New Zealand and tentatively recognised in Czechoslovakia, the Northern Pacific, the Gulf of Mexico, Japan and France. Noël⁴ has described further palaeomagnetic results from two Swedish varved-clay cores as accurately tracing the annual behaviour of the geomagnetic event and delimiting its initiation and termination. Recognition of the event in lavas and deep sea, continental shelf, varved-clay and lake sediments has been cited as confirming the reality of the event. That previous research, particularly in Sweden, has concentrated on palaeomagnetic measurements at numerous, different late Weichselian localities. Because of the difficulties in correlating between localities, interpreting palaeomagnetic records in coarse grained sediments and deciphering coring and sedimentological complexities in single cores, we have instead

studied one carefully selected site in detail. We have come to the conclusion that Swedish late Weichselian palaeomagnetic data do not record a reversal or excursion of the geomagnetic field, but demonstrate that the Swedish geomagnetic field has been of normal polarity since 13,000 yr BP. The Swedish late Weichselian reversal had thus become an example of Watkins⁵ "reinforcement syndrome".

We have analysed 408 subsamples from two main cores collected at Björkeröds Mosse on Mt Kullen in north-western Scania in southern Sweden (56.2°N, 12.6°E). Björkeröds Mosse was chosen because it lies in the area considered to be the earliest ice-free part of Sweden⁶; its stratigraphy, vegetational and soil developments have been studied in detail^{7,8}; it had a high rate of deposition of organic sediments; and its chronology is well known from both pollen and radiocarbon dating⁷. The sediments from Björkeröds Mosse span the supposed age of the late Weichselian geomagnetic reversal of 12,400 or 12,100 yr BP. Ten sections of 'D'-shaped core, each 1 m in length, were obtained using a Jowsey sampler⁹. The natural remanent magnetisation (NRM) of the 408 subsamples was measured on a 'Digico' magnetometer¹⁰. Twenty-two pilot samples were partially a.c. demagnetised stepwise in increasing peak fields. A peak alternating field of 100 oersted was selected to eliminate viscous components. The remaining 386 subsamples were cleaned at this field strength. NRM, cleaned declination and inclination, cleaned intensity, and low field reversible susceptibility of core III (1975) are shown in Fig. 1.

We interpret the palaeomagnetic directions as follows. In general, the geomagnetic field close to the time of deposition is recorded by the palaeomagnetic remanence. At a few lithologically distinct horizons, however, the magnetic remanence has been controlled by other processes. The scattered or offset declinations and inclinations in the laminated deposits of layer 1 at depths of 615, 520 and 440 cm (Fig. 1) occur in coarser grained sediments and are due to rough alignment in higher energy deposition environment, rather than to idealised alignment along the ancient magnetic field direction. The single, reversed inclination at a depth of 338 cm (Fig. 1) falls in a narrow dark layer about 2 cm thick. A dark layer at a similar stratigraphic level in Björkeröds Mosse has been reported by Mörner in core B892 to be "well established" as a geomagnetic excursion and to correspond to his Fjards interstadial. The layer

Fig. 1 Data from Björkeröds Mosse core III (1975). ○, NRM; ●, partial demagnetisation at 100 oersted. Crosses indicate end of core sections. Mean declinations were set to zero.



in our core III (1975) is anomalous as, (1) its base is markedly (our results, in preparation) crenelated, (2) it does not occur in two other duplicate cores, and (3) the remanence is unusually stable as well as in a distinct direction. Its pollen spectrum and sediment chemistry correspond to the till which forms the banks of the lake. We prefer to interpret this intermediate palaeomagnetic direction and Mörner's¹¹ in core B892 to be due to slumping rather than a true reflection of the geomagnetic field. The remaining great majority of palaeomagnetic directions are all of normal polarity and are taken to indicate that the geomagnetic field was of normal polarity from 13,000 to 11,000 yr BP in Sweden.

The retention of aberrant magnetic directions and the between-core repeatability (our results, in preparation) of cleaned remanent intensity indicate that these normal polarity late Weichselian palaeomagnetic directions are almost contemporary with deposition and are unlikely to have been caused by later remagnetisation. Our data suggest previously reported reversed palaeomagnetic directions¹⁻⁴ are not reliable indicators of the ancient geomagnetic field, but have been distorted by mechanical sedimentation processes, slumping or weathering. Alternatively, but less likely, the reversed directions had been dated inaccurately. We suggest the proliferation of unusual palaeomagnetic directions in Scandinavia around 12,000 yr BP is a reflection of changing climatic conditions. In many localities the fluctuations of climate produced sediments of very variable mechanical properties, particularly at times of periglacial activity, which were poor recorders of the ambient magnetic field direction.

Using geomagnetic events in the Swedish late Weichselian is thus of doubtful value. Furthermore, reversals or excursions of the geomagnetic field from other parts of the world around this time should not be dated or correlated with the earlier Swedish results.

Watkins¹² warnings on "the special problems posed by the search for short events" have unfortunately not been heeded. We endorse his comments¹² and on the basis of our work on the Swedish late Weichselian propose three minimum requirements for the recognition of geomagnetic excursions in recent sediments: (1) both magnetic declination and inclination changes should be repeatable to within 20° in at least two sections or cores at the same site; (2) results should only be taken from macroscopically homogeneous lithologies with an average grain size below 62.5 µm; and (3) magnetic directions should be stable under alternating field cleaning with peak field ≥ 100 oersted.

Application of these criteria would prevent many spurious palaeomagnetic directions being interpreted as evidence for unusual geomagnetic behaviour. It would reduce the large number of excursions and events deduced from Brunhes sediments to a small total comparable to that recognised in igneous material¹³. It would also reduce the difficult dating problems arising from attempts to correlate recent excursions, dispel speculation¹¹ on very rapid changes in the main dipole field and enable geomagnetic excursions to be considered again as chronological indicators.

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January-thaw singularity and wave climates along the Eastern coast of the USA

METEOROLOGICAL singularities—recurrent weather phenomena at or near specific calendar dates¹—have been reported throughout the Northern Hemisphere. Although not fully recognised as real phenomena by most meteorologists, singularities continue to merit the attention of some investigators. Although the general relationship between wind fields over open oceans and generation of waves by wind are well known, there are no reports in the literature on coastal wave climates and atmospheric singularities. I have observed that extreme frequencies of surf from directions more typical of the summer season occur at the time of the 'January-thaw' meteorological singularity (January 20-23). Here I present observations of wave climates from Florida to Rhode Island at the time of the January thaw.

Slocum² recorded an anomalous warm spell during January 20-23, between 1872 and 1921. Subsequently, Wahl³ demonstrated the existence of the January thaw in New England for the period 1873-1952 and Frederick⁴ reported on its extensive geographical distribution between 1927 and 1956. Wahl³ also noted that at the time of the January thaw the ridge-and-trough positioning over eastern North America differed markedly from its positioning before afterwards; specifically, during the January thaw there is a high pressure cell off the east coast of the USA, and a trough runs through the midwest, whereas immediately following the thaw, the positions of the ridge and trough are reversed. Anticyclonic singularities have been reported for Europe (January 18-24) by Brooks⁵ and Japan (January 24) by Maejima⁶.

Duquet⁷ has attributed the January thaw to an adjustment of the planetary circulation occurring in early or mid January. Wahl⁸ has noted that the January thaw is most pronounced during low-index Januaries. Significant changes in the general circulation of the atmosphere at the time of the January thaw are well documented. These changes should appear as a singularity in coastal wave-climate statistics.

Surf-height and direction statistics for years falling within a period of general low index⁹ were obtained from the Co-operative Surf Observation Program (COSOP) for Point Judith, Rhode Island (1957-1965); Atlantic City, New Jersey (1955-1964); Ocean City, Maryland (1955-1970); Virginia Beach, Virginia (1954-1969); Oak Island, North Carolina (1955-1965); and Hillsboro Inlet, Florida (1955-1965), (Table 1). Daily and 3-d running means of surf heights were examined but did not show any anomalous characteristics at the time of the singularity; however, at the time of the January-thaw singularity, the directions of approaching surf along the Atlantic coast are atypical of winter. The magnitude of the departure from winter conditions to surf conditions more typical of summer are demonstrated by the occurrence of extreme frequencies for various surf directions during the period of the singularity (Table 1). Although Wahl³ has shown that the pressure field characteristic of the singularity is present on January 20, the maxima and minima of frequencies for each surf direction generally occur around January 23. This apparent lag may be attributable to the required time of wave generation and travel to the coast.

Table 1 Daily and 3-day extremes of surf directions along the US eastern coast during the January thaw

Station and surf direction	January thaw		January daily mean (%)	Month of daily	
	Daily extreme (Date) (%)	3-day mean extreme (mid-date)		maximum (% frequencies)	minimum
Point Judith, Rhode Island					
SE	38 (23rd)*	44 (22nd)*	55	May (65)	Sep (47)
SW	56 (22nd)§	43 (22nd)§	27	Aug (28)	May (19)
Atlantic City, New Jersey					
NE	0 (23rd)*	5 (23rd)	9	Dec (16)	Aug (1)
SE	57 (23rd)	63 (23rd)	77	Jul (84)	Mar (67)
S	19 (23rd)†	12 (23rd)†	3	May (7)	Oct (1)
Ocean City, Maryland					
NE	15 (21st)	18 (22nd)	23	Dec (30)	Jul (6)
E	28 (21st)	37 (22nd)	42	Dec (44)	Jul (26)
SE	52 (23rd)†	43 (22nd)†	30	Jul (58)	Dec (25)
Virginia Beach, Virginia					
NE	16 (23rd)*	26 (22nd)	39	Jan (39)	Jul (8)
SE	37 (23rd)†	28 (22nd)†	15	Jul (55)	Jan (15)
Oak Island, North Carolina					
SE	0 (22nd)	4 (21st)	5	May (13)	Jul (1)
S	74 (22nd)	69 (22nd)†	57	Oct (66)	Jun (52)
SW	26 (22nd)	25 (22nd)*	37	Apr (46)	Oct (24)
Hillsboro Inlet, Florida					
NE	0 (24th)*	5 (23rd)*	21	Jan (21)	Jul (1)
E	24 (23rd)†	34 (23rd)	42	Apr (59)	Jan (42)
SE	47 (24th)§	42 (23rd)§	15	Jul (24)	Oct (7)

*Minimum value for month of January.

†Maximum value for month of January.

‡Minimum value for year.

§Maximum value for year.

At Ocean City, Virginia Beach, and Hillsboro Inlet, surf from the south-east dominates. At Atlantic City extreme high frequencies of south surf occur, and at Point Judith surf from the south-west dominates. On Oak Island southerly and south-westerly surf dominates, and at Hillsboro Inlet north-easterly and south-easterly surf predominates. The hypothesis enunciated here is not supported only at Atlantic City where the 1% level-of-significance criterion was not met.

Duquet⁷ has suggested that the January thaw is a manifestation of "an adjustment of the planetary circulation from what might be called an early winter stage to a late winter stage . . ." The statistics of south-easterly surf at Hillsboro Inlet support the season-transition concept. The period of October 1–January 20 is characterised by a mean frequency of south-easterly surf of about 9%. During the period January 20–end March, the mean frequency of surf from the south-east is 23%. Thus, the time progression of south-easterly surf at Hillsboro Inlet takes the form of step functions rather than of a monotonic sequence.

Coastal wave climates are traditionally viewed as a simple annual cycle between summer and winter conditions, and definition within the annual cycle is not generally recognised. If other atmospheric singularities are found to mark simultaneous changes in wave climates, then such information would have immediate application to coastal planning in general, and coastal engineering in particular. Further investigation of coastal wave climates should answer this question and may further clarify the many unanswered questions about atmospheric singularities.

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Middle Pleistocene stratigraphy in southern East Anglia

A STUDY of Quaternary deposits in south-east Suffolk revealed a complex, and hitherto unrecognised Middle Pleistocene stratigraphic sequence¹. The probable ages of the units in the sequence are, from the base upwards, river sands and gravels of Beestonian age, a rubified palaeosol of Cromerian age, and a periglacial palaeosol, loess, glacial fluvial gravel, and till of Anglian age (Table 1). A preliminary survey of the Quaternary deposits in Essex has revealed a similar sequence. We here outline the main characteristics of this sequence, which has now been recognised over some 2,000 km² in southern East Anglia, and discuss its palaeo-environmental significance.

The Beestonian sands and gravels are the most widespread Pleistocene deposit in the region. They form a body which trends south-west–north-east across the area (Fig. 1) and reaches a maximum observed thickness of 30 m. They are characteristically though not invariably a whitish colour, and are composed predominantly of flint and quartzite with small percentages of other rocks such as chert, rhyolite and tuff². Far travelled materials such as Bunter quartzite, chert and volcanic rocks show that much of the deposit has been transported into the region, and the absence of non-durable material such as chalk, London Clay mudballs, and Jurassic shells and shale from the main body of the deposit suggests that the bulk of the material has been subjected to prolonged fluvial abrasion. North-eastward dipping large scale and small scale cross-set structures with occasional silt lenses indicate that the material was deposited as bars and dunes by a river with an irregular regime and a north-eastward direction of flow. Intraformational ice-wedge casts, vein-ice casts, and segregated ice accommodation structures are developed throughout the deposit, demonstrating that the fluvial processes operated in a periglacial environment.

The height range of the surface of the deposit (Fig. 2) suggests that it is composite in origin, and may represent a series of river terraces formed at progressively lower levels as the river migrated south-eastwards. Considered in a regional context this body of sand and gravel probably

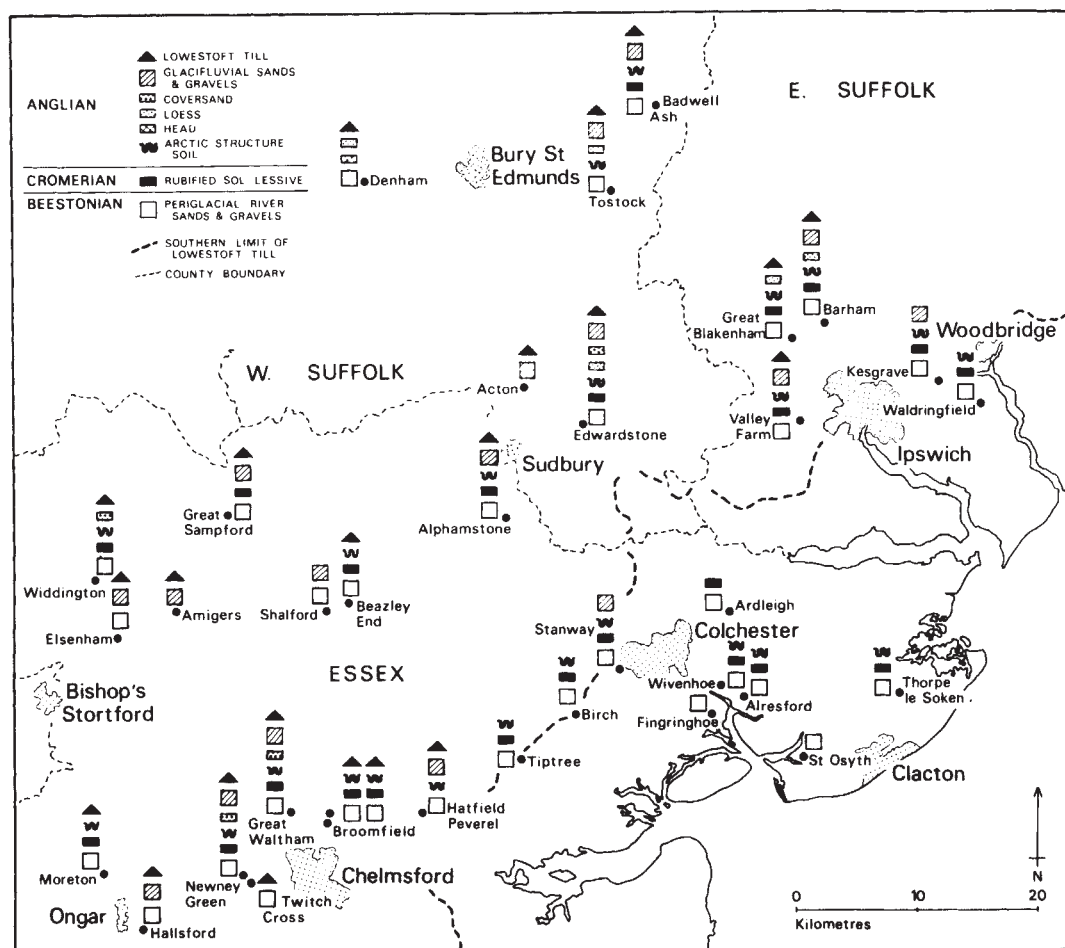


Fig. 1 Middle Pleistocene stratigraphy in southern East Anglia.

represents the debris of a much larger Thames drainage system, operating with a load and regime controlled by periglacial conditions.

These Beestonian sands and gravels were included by Prestwich³ in his Westleton Beds which he considered to be marine in origin and separate from the glacial gravels. In contrast, Clayton⁴ regarded all the sands and gravels of the Chelmsford and Harlow regions as glacial outwash, naming them the Danbury and Chelmsford Gravels, though Solomon⁴ pointed out that the heavy mineral assemblage indicated that there were two separate units, one of which could not be associated with the local till. Bristow and Cox⁵ also regarded these two gravels as glacial outwash but proposed that they both be called the Chelmsford Gravels.

The Cromerian palaeosol is a rubified *sol lessivé modaux*⁶, and is developed on the surface of the Beestonian sands and gravels. Usually this soil is truncated, so that only the alluvial horizon survives in the form of a reddened zone associated with cutans, clay enrichment and iron enrichment. Soils of this type are produced in a humid warm

temperate climate^{7,8}, and as such reflect stable soil conditions which existed beneath the Cromerian mixed oak forest⁹.

The Anglian palaeosol is an arctic structure soil¹⁰, and is identified by involutions, frost cracks, and epigenetic ice-wedge casts which disturb the horization of the rubified *sol lessivé* and the primary structures in the upper part of the Beestonian sands and gravels. The loess that is developed in south-east Suffolk is represented by coversand in Essex (Fig. 1). The stratigraphic equivalence is demonstrated by a diagnostic heavy mineral assemblage (J. A. Catt, personal communication). Where both deposits exist at a site the coversand is uppermost. Their association with the arctic structure soil is shown by the fact that both deposits mix with the involutions and rest on the periglacial soil surface. At locations where the arctic structure soil has not been subsequently buried by the till, only the basal part of the ground ice structures remain, and the upper parts have been disturbed by younger periglacial activity.

The Anglian glacifluvial sands and gravels succeed the

Table 1 Middle Pleistocene stratigraphy and palaeoenvironments in southern East Anglia

Stage name	Formation name in south-east Suffolk	Environment	Sediments and palaeosols in Essex and west Suffolk
Anglian	Lowestoft Till	Glacial	Till (Lowestoft Till ^{11,12})
	Barham Sands and Gravels	Glacifluvial	Sands and Gravels
	Barham Loess	Periglacial	{ Loess and Coversand Head Arctic Structure Soil
	Barham Arctic Structure Soil		
Cromerian	Valley Farm Rubified <i>Sol Lessivé</i>	Humid, warm temperate	Rubified <i>sol lessivé</i>
Beestonian	Kesgrave Sands and Gravels	Periglacial	Sands and Gravels (Essex White Ballast ^{3,11})

arctic structure soil. This deposit is only occasionally present beneath the till (Fig. 1), and beyond the till margin it is restricted to channels cut in underlying deposits. The base of the deposit is characterised by a pebble lag, but the main body consists of pale brown, well sorted sand and gravel composed predominantly of locally derived flint and quartzite (from the Beestonian sand and gravel) and a small component of glacially derived non-durable rocks. Primary sedimentary structures consist of small channels, plane beds, and small scale, tabular cross-sets which dip predominantly south-eastwards in Suffolk, and indicate that the deposit formed as dunes in shallow, braided rivers which drained away from the Lowestoft Till ice sheet in directions normal to the ice margin. Ground ice structures are absent from this deposit, and suggest that sedimentation rates were more rapid than the rate at which permafrost could develop. This deposit includes the upper part of the Chelmsford gravels⁴.

Lowestoft Till of Anglian age^{11,12} forms the upper part of the succession, and several of the sites have been studied by Perrin *et al.*¹². At many locations the till rests directly on the undisturbed palaeosols or the wind-blown sediments. This indicates that over parts of southern East Anglia, glacial erosion did not occur, and probably suggests that flow tills protected the existing deposits before they were overridden by glacier ice.

The palaeoenvironmental significance of the Middle Pleistocene stratigraphic sequence in southern East Anglia can be summarised as follows:

(1) Two separate sand and gravel bodies can be recognised. The Beestonian river deposit is extensive throughout the region. The Anglian glacial deposit is thin and localised. The earlier deposit is related to a north-eastward system of drainage. The present-day south-eastward and southward drainage patterns in the region were initiated during the Anglian glaciation with the deposition of the proglacial outwash.

(2) Downcutting and migration by the Thames drainage system began during the Beestonian periglacial stage, and by the time of the Cromerian Interglacial, the river must have adopted a route south and east of a line from Chelmsford to Colchester and Woodbridge (Fig. 2).

(3) The Cromerian rubified *sol lessivé* is developed widely across southern East Anglia. Where it is buried beneath Anglian glacial sediments it forms a clear stratigraphic horizon. Where it remains at the surface it forms the parent material for present-day soils which consequently have a reddened appearance.

(4) The Anglian arctic structure soil is developed across the region in association with loess and coversand. These aeolian deposits differ from Devensian aeolian sediments in their heavy mineral composition.

(5) Glacial erosion of pre-existing sediments and soils is negligible over large parts of the glacierised area of southern East Anglia.

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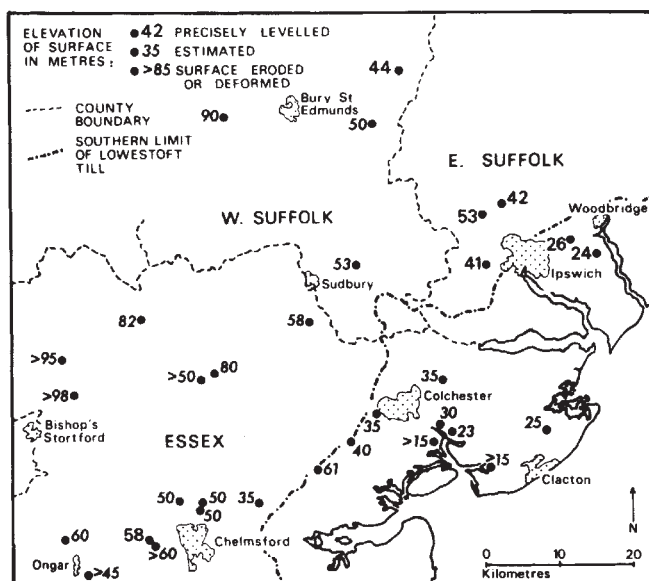
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Fig. 2 Elevations of surface of Beestonian sands and gravels. Locations are indicated on Fig. 1.



Oldest recorded *in situ* tracheids

RECENT critical reviews¹ have suggested that evidence from microfossils² (that is spores with triradiate marks, sheets of "cells" or tubes with tracheid-like thickenings) should not be considered sufficient to demonstrate the existence of vascular plants in Silurian times; the only acceptable

Fig. 1 Terminal globose sporangium ($\times 18$).



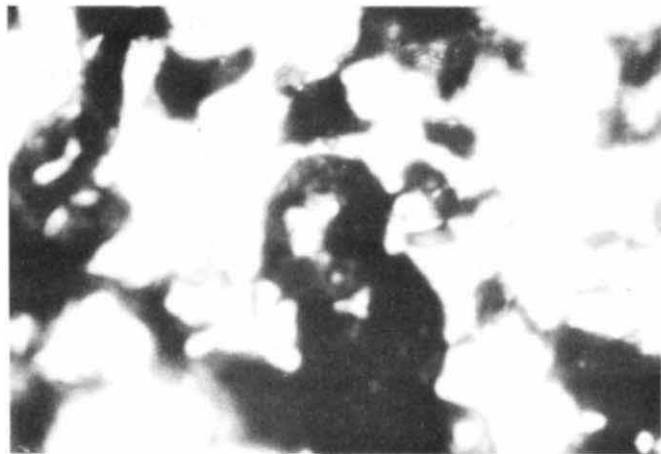


Fig. 2 Two spores with gaping trilete marks isolated on film pull and photographed using infrared film ($\times 750$).

evidence being megafossils with tracheids *in situ*. Such was the evidence presented by Lang³ when he described smooth axes from the Downton Series (Pridolian $\epsilon\beta_2$) of the Welsh Borderland. These vascularised axes were found in association with two species of *Cooksonia*, very simple plants with smooth forking axes terminating in globose sporangia which contained spores bearing trilete marks. Megafossils morphologically similar to *Cooksonia* have been described from strata of similar age throughout the world, but the Lang specimens are the only ones with unequivocal tracheids and thus have hitherto been considered the earliest vascular plants. We report here the occurrence of a vascular plant in the slightly older Whitcliffian strata (Ludlow Series) of South Wales.

Our investigations have centred on a lower Whitcliffian flora (uppermost Ludlow Series $\equiv$ Kopaninan $\epsilon\beta_1$) from the Capel Horeb Quarry, Powys (SN 844323); the age of the plant-bearing Roman Camp Beds being based on faunal⁴ and microfossil evidence (personal communication from K. Dorning). Among smooth often dichotomously branching carbonaceous axes are a few *Cooksonia*-type sporangia (Fig. 1) some of which contain spores with trilete marks (Fig. 2). A central longitudinal line visible in some of the axes is presumed to represent conducting tissue⁵, although until the study reported here tracheids have not been isolated from this region. Regular transverse banding reminiscent of tracheidal pitting (Fig. 3) is often observed, but there is a possibility that such a pattern has been produced by the

Fig. 3 Possible tracheid showing regular transverse banding and a single longitudinal wall ($\times 416$).

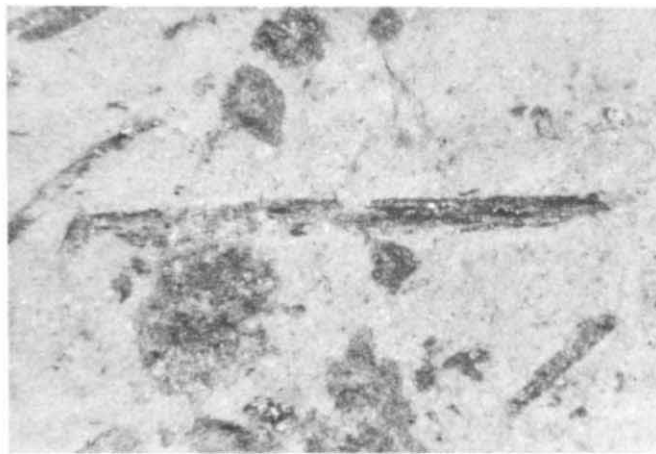


Fig. 4 Unbranched carbonaceous stem from which tracheids in Fig. 5 were isolated ($\times 7$).

regular fracturing of the carbonaceous residues. This explanation, however, cannot be applied to the short lengths of tracheids recovered on a cellulose acetate film pull from an unbranched axis 10 mm long and 1 mm wide (Fig. 4). Figure 5 shows two, possibly three adjacent incomplete tracheids, partly masked by a darker, more opaque, non-cellular layer. The longitudinal walls are $7.5\ \mu\text{m}$ apart in the longest element and the horizontal bars are, on average, $5\ \mu\text{m}$ apart. Although we are reluctant to draw any conclusions on the type of pitting from such fragmentary compression fossils, there is some resemblance to the annular thickening described by Lang in his Downtonian specimens.

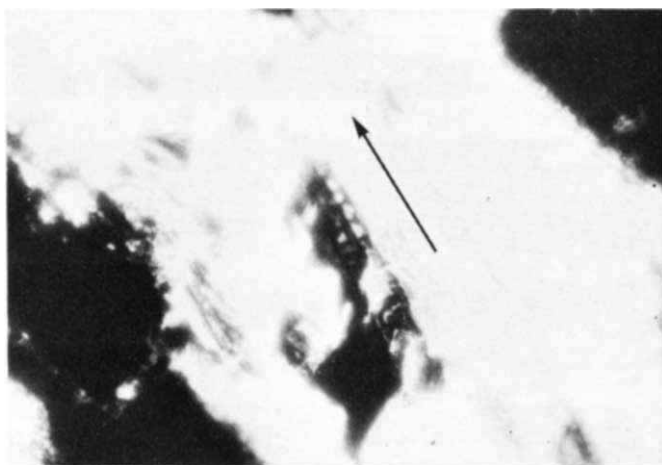


Fig. 5 Fragments of two adjacent tracheids isolated on a film pull and photographed using infrared film. Arrow indicates longitudinal axis of stem ($\times 416$).

Our findings therefore indicate the presence of vascular plants, most probably of *Cooksonia*-type organisation, in the upper part of the Ludlow Series, slightly older than the Downtonian vascularised axes of Lang.

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Wild bank voles (*Clethrionomys glareolus*) are possibly a natural reservoir of campylobacters (microaerophilic vibrios)

STRAINS of *Campylobacter*, a genus of microaerophilic bacteria, have been isolated by various workers from the gut and faeces of several animal species including man¹, but as far as we know, there have been no reports of their isolation from rodents. The continuing importance of diseases of farm animals involving campylobacters, for example infectious infertility and sporadic abortion in cattle², abortion in sheep³ and dysentery in pigs³, and the paucity of information on the source of outbreaks of some of these diseases prompted us to investigate whether campylobacters were present in the faeces of bank voles (*Clethrionomys glareolus*), short tailed field voles (*Microtus agrestis*) and long tailed field mice (*Apodemus sylvaticus*) from their natural habitat. These species are common throughout mainland Great Britain⁴ and were being trapped as part of an investigation into the distribution of pathogenic bacteria and viruses in small wild rodents. Bank voles and field mice are commonly found in woodland and scrub, and short tailed field voles are found in grassland, therefore all three species might provide a source of infection for domestic animals. We report here that bank voles carry campylobacters.

At intervals of 6 weeks during a period of 3 months bank voles and field mice were trapped in an area of mixed broadleaved woodland in Berkshire, and short tailed field voles were trapped in an area of well established grassland in Hampshire. The animals were caught in Longworth traps⁵ arranged in a squared grid of 3,600 m² in each trapping area. Two traps were placed at each point, with an interstation distance of 10 m. A faecal pellet voided by each animal was placed in 5 ml of quarter strength Ringer's solution in a 0.5-ounce McCartney bottle, and maintained at 18–20 °C for not more than 3 h before selective culture for campylobacters on blood agar by a filtration method¹. Isolation plates were examined after incubation in 30% (v/v) CO₂ in air for 4 d at 37 °C. If colonies of *Campylobacter* were not detected, the plates were reincubated and examined after a further 10 d.

We isolated bacteria which we identified as *Campylobacter* from the faeces of 10 out of 13 bank voles, but not from the faeces of any of 12 field mice or any of 17 short tailed field voles examined. *Campylobacter* isolates from bank voles were motile, Gram-negative, slender curved rods with a predominance of coccal forms in old (5 d) cultures. On blood agar after incubation for 3 d at 37 °C colonies were irregular, tending to spread. Each colony had an entire edge and was light grey with a diameter of 2–4 mm. All isolates produced catalase and reduced nitrate to nitrite, but did not produce acid in media containing glucose, grow in nutrient gelatin or haemolyse blood. They produced H₂S detectable by lead acetate paper only when grown in a medium with added cysteine⁶. Growth did not occur, or was very slow, in media containing 1% (v/v) glycine and in media incubated either in air or in anaerobic conditions. By these criteria our isolates resemble *C. fetus* ssp. *venerealis* biotype *intermedius*⁶, a resemblance which is interesting since campylobacters with these characteristics have been recognised as a cause of infectious infertility in cattle⁷.

Our work shows that animals hitherto not considered in studies on the epidemiology of diseases of domestic animals carry campylobacters and may provide a reservoir of infection. Furthermore, it is possible that *Clethrionomys glareolus* might provide what is much needed for research into campylobacter infections—a laboratory model for pathogenicity studies.

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Population density affecting adult shell size of snail *Cepaea nemoralis* L.

THOMAS *et al.*^{1,2} have questioned the ability of molluscs to limit their population size by self inhibition at high densities. They suggest that plant metabolites may have caused the reduction in growth and fecundity of the aquatic snail *Biomphalaria glabrata* Say ascribed to crowding in previous studies^{3,4}. Field evidence for density-dependent regulation is limited. Yom-Tov⁵ found that the fecundity of the desert snail *Trochoidea seetzeni* Pfeiffer was adversely affected by population density; he considered that either self-inhibition or nutritional differences were responsible. While studying the population dynamics and energetics of the land snail *Cepaea nemoralis* L., we have found evidence for density-dependent effects that cannot readily be explained by resource limitation. In samples of *C. nemoralis* collected in 1968 to study shell polymorphism (M. A. Palles-Clark, unpublished) a negative correlation was noticed between adult shell diameter and sample size ($r = -0.60$, $P < 0.001$). Snails were collected from chalk grassland between Beacon Hill and Round Down, West Sussex. A more detailed study carried out in the same area during the summer of 1973 confirmed this effect. The density of adult *C. nemoralis* was measured by mark-recapture at nine 20 × 20 m sites, situated 100–600 m apart. Each site was sampled in May, June and August. As there were insufficient recaptures to use multiple recapture methods for all sites, adult population densities were estimated for the second sampling occasion using the Lincoln index.

Measurement of adult shell diameter showed a 6–9% decrease in adult size associated with an increase in density from 0.5 to 5.5 adults per m² (Fig. 1). The pattern of population density was patchy but similar to that found in 1968. There were no simple relationships between density and geographical features, for example altitude or aspect. There were slight differences in shell colour and banding morph frequencies between the sites but these did not relate to differences in adult density or shell size.

Figure 1 also shows changes in mean shell diameter with season, reflecting a change in age structure of the adult population. Separate values of shell diameter are given for May, when the collections were mostly of old adults, surviving from previous years, and for August, when new adults (with shiny shells, periostracum intact) predominated. The August results exclude snails captured and marked in either May or June. Snails were considered adult when a lip had been formed at the mouth of the shell; after lip formation there is no further increase in shell diameter. Regression lines drawn through each set of data had significantly different slopes ($P < 0.02$), the change in slope indicating that recruitment to the low density populations increased mean adult size, whereas recruitment to the high density populations had the opposite effect. Long term

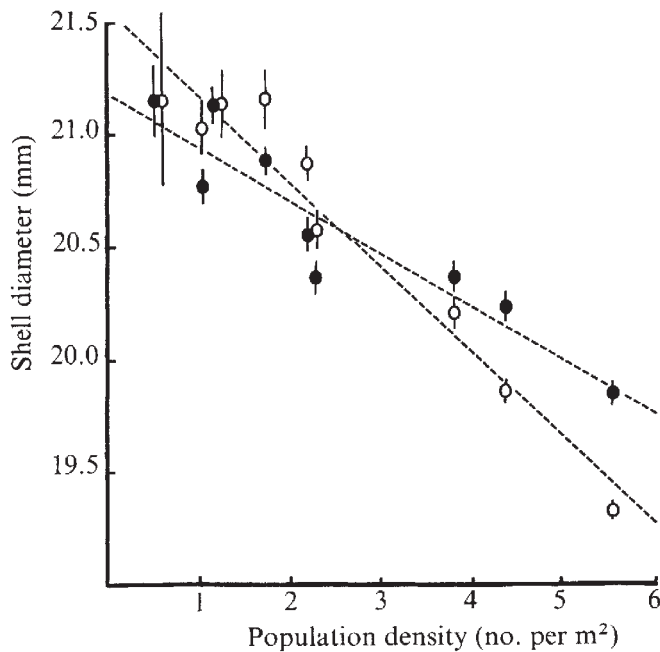


Fig. 1 Relationship of mean adult shell diameter ($\pm$ s.e.) and adult population density. ●, Samples collected on May 23, $Y = 21.19 - 0.238X$ (s.e. slope = 0.038); ○, samples collected on August 7, $Y = 21.56 - 0.382X$ (s.e. slope = 0.034).

studies at an adjacent site did not show any significant changes in adult shell diameter over autumn and winter. It therefore seems that the differences between new and old adults are a function of the conditions experienced by juveniles, rather than selection pressures operating on adults. Unfortunately our field data do not enable us to separate the effects of juvenile and adult population densities on juvenile growth rates, nor to determine the time when growth is most affected.

The decrease in shell diameter with increasing density has a marked effect on body weight, reducing it by 14–31% (values determined from log–log regressions of weight and size for new and old adults⁶). Wolda's studies⁷, in field conditions and in the laboratory, indicate that fecundity would be similarly reduced. His regressions relating shell diameter to clutch size and oviposition frequency for *C. nemoralis* predict a 22% decrease in annual egg production when adult shell diameter changes from 21.2 mm to 19.3 mm. Overwinter survival could also be affected: Cook and O'Donald⁸ found that smaller adult *C. nemoralis* from a Pyrenean population were at a selective disadvantage when kept in experimental conditions producing high mortalities during dormancy.

A similar relationship between shell size and population density was detected by further analysis of Wolda's⁸ field data. When adult shell diameter was plotted against density index the regression slope was negative and significantly different from zero ($P < 0.001$, combined data from east-west and north-south roads). Wolda's sampling sites were distributed over a total area similar to ours (about 0.5 km²). Over larger areas, the variation in other factors affecting shell size, for example calcium availability, would be increased; consequently we would not expect the effect of population density to be so marked.

There was no indication that *C. nemoralis* at our study area was limited by either food quantity or quality. Point quadrat surveys of the nine sites did not show any correlation between population density and the vegetation biomass, nor between density and the proportion of palatable plants in the standing crop (R.A.D.C., P.W. and D. I. Morgan-Huws, unpublished). The feeding of *C. nemoralis* was studied in detail for a population with a

density of five adults per m² in June 1973, and a mean annual biomass (March 1973–March 1974) of 1.03 g m⁻² for adults and juveniles. It was estimated¹⁰ that this population consumed 3.7% of the above-ground production of herbs, 0.6% of that of grasses, and 0.91% of the total above-ground primary production. Preferred foods (dead herbs) were available at all times of the year. Calcium deficiencies were most unlikely: at all sites pieces of chalk were present at or near the soil surface. Shells of dead snails provided an alternative supply.

We therefore conclude that interactions between snails, either chemical or behavioural, were responsible for slowing juvenile growth rates in high density populations, resulting in smaller adults, and thus affecting the birth rate in succeeding years. It is likely that the changes in adult shell size that occurred in Wolda's field⁹ and experimental¹¹ populations of *C. nemoralis* were caused by similar density-dependent effects. Laboratory studies are in progress to determine the effect of density on juvenile growth in experimental conditions. Initial results show that the presence of mucus trails of other snails inhibits activity, suggesting a possible mechanism for the effects observed in the field.

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Overdominance and U-shaped gene frequency distributions

A GENERAL pattern that emerges from electrophoretic analyses of protein variation in natural populations is that alleles with intermediate frequencies are rare. This results, graphically, in a U-shaped frequency distribution when both allele frequencies from a two-allele locus are plotted. Supporters of the neutralist theory of population variation believe that most protein polymorphisms found in nature are the result of the random drift of neutral mutations and argue that a U-shaped distribution is expected for the case $4Ns < 1$, where N is the population size of diploid organisms and s is the average selection coefficient. We show here that, under certain assumptions, a U-shaped frequency distribution also obtains when $4Ns > 1$. From this we conclude that the analysis of allele frequency distributions (or, equivalently, relative contributions to heterozygosity) does not provide a sensitive means of resolving the neutralist and selectionist controversy.

In the textbook model of an overdominant polymorphism, two alleles, A and B, segregate at a locus, the frequency of allele A indicated by $\hat{p}$ and the genotypes AA, AB and BB have relative fitnesses $1-s_1$, 1 and $1-s_2$, respectively, where

$0 < s < 1$. It is easily shown that the equilibrium frequency of A, denoted $\hat{p}$, is $s_2/(s_1+s_2)$. Various biochemical, physiological, genetic and ecological mechanisms can account for the relative superiority of the heterozygote. Our approach will specify the distribution of $\hat{p}$, given certain assumptions about the distributions of s_1 and s_2 . We begin by postulating that s_1 and s_2 are independent, and will discuss the possibility of positive or negative correlation later.

The probability distribution of selection coefficients, $g(s)$, defined on the interval $0 < s < 1$, gives the probability that a selection coefficient will lie between s and $(s+ds)$. We assume a single parameter family of such distributions, where b is a parameter that reflects different biological reality (see below)

$$g(s) = \frac{1}{b} s^{(1/b)-1} \quad (1)$$

We now seek the probability distribution for equilibrium gene frequency, $h(\hat{p})$, given that both s_1 and s_2 are distributed according to equation (1). We define two new variables

$$x_1 = s_1/(s_1+s_2) \quad (2a)$$

$$x_2 = s_1+s_2 \quad (2b)$$

Solving for s_1 and s_2 gives

$$s_1 = x_1 x_2 \quad (3a)$$

$$s_2 = x_2(1-x_1) \quad (3b)$$

The Jacobian of transformation (3), that is, the determinate of $\partial s_i / \partial x_j$, is x_2 , thus the joint probability distribution of x_1 and x_2 is

$$H(x_1, x_2) = \left[\frac{1}{b} (x_1 x_2)^{(1/b)-1} \right] \left[\frac{1}{b} (x_2(1-x_1))^{(1/b)-1} \right] x_2 \quad (4)$$

Since x_1 and x_2 can be separated

$$H(x_1, x_2) = \frac{x_2^{(2/b)-1}}{b^2} (x_1(1-x_1))^{(1/b)-1} \quad (5)$$

It is then possible to obtain the marginal probability distribution of x_1 , $h(x_1)$, by integrating over x_2 in the domain of the distribution of distribution function H ($0 \leq x_2 \leq 1$, $0 \leq x_1 \leq \min(1/x_2, 1 - [1/x_2])$), yielding

$$h(x_1) = \begin{cases} x_1^{(1/b)-1} (1-x_1)^{[(1/b)-1]/(2b)}, & 0 \leq x_1 \leq 0.5 \quad (6a) \\ x_1^{[(1/b)-1]/(2b)} (1-x_1)^{(1/b)-1}, & 0.5 \leq x_1 \leq 1 \quad (6b) \end{cases}$$

This distribution, symmetrical about 0.5, gives the probability of $1 - \hat{p} (= \hat{p})$ if s_1 and s_2 are taken from probability distribution equation (1). Functions $g(s)$ are plotted in Fig. 1A for different values of b , with the corresponding $h(\hat{p})$ distributions plotted in Fig. 1B. The question remaining is which, if any, values of b correspond to biological reality.

Although a fairly high percentage of newly arisen mutations may be lethal, most of the alleles persisting at a locus in a natural population are not seriously deleterious as homozygotes: really deleterious genes will be eliminated soon after they appear. Thus it is reasonable to postulate that homozygotes that persist in a population will have fitness nearly equal to the heterozygote and that the mode of the distribution $g(s)$ will be at $s = 0$, with the probability of larger s falling off rapidly with increasing s . This corresponds to large values of b , which give U-shaped gene frequency distributions.

As mentioned, we have assumed that s values are uncorrelated. A negative correlation, which pairs large and small s values,

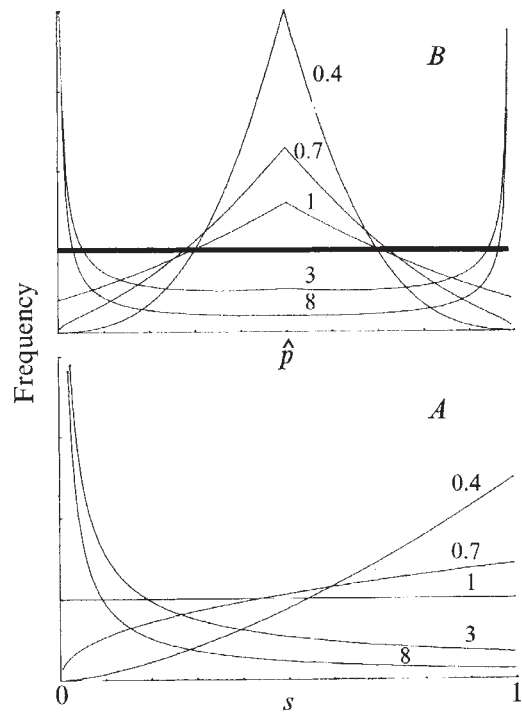


Fig. 1 A, Some $g(s)$ distributions for different values of the parameter b , the numerical value of which is given above the line. B, Some $h(\hat{p})$ distributions for the same five values of parameter b . The heavy line is a hypothetical distribution for which all values of $\hat{p}$ are equally likely.

seems to us absurd; it would, however, increase the tendency towards U-shaped gene frequency distributions. Positive correlations between s values work against U-shaped distributions. A likely source of such a positive correlation is a situation where the selection coefficients for the homozygotes at some 'important' loci tend to be high, whereas those for other 'less important' loci tend to be low. We admit that some loci are more essential than others, but alleles persisting in natural populations at such relatively essential loci cannot deviate far from optimal functioning before the s values become large. Conversely, populations seem to tolerate a much broader range of function at loci which *a priori* are less critical metabolically. For example, an $s = 0.5$ might correspond to a very slight biochemical change in a regulatory enzyme on the one hand, and to a virtual absence of function of a coat colour gene on the other. We feel therefore, that it is incorrect to equate physiological or developmental importance with the effect of a locus on fitness. Rather, it is reasonable that the s distributions for all types of loci are the same and that our assumption that the individual s values at a locus are uncorrelated is warranted.

This result is not solely dependent on equation (1). We have used other single parameter probability distributions for $g(s)$. One family of functions was

$$g(s) = b(1-s^b)/(b+1) \quad (7)$$

We were not able to obtain $h(p)$ analytically for this function, so we used numerical integration on a computer. Distributions of $g(s)$ which were concave towards the origin (corresponding to values of b in equation (7) less than 1) gave U-shaped distributions for the equilibrium gene frequency. In addition, we treated other $g(s)$ distributions in a Monte Carlo fashion and arrived at the same conclusion. If the natural distribution of selection coefficients is concave towards the origin, then U-shaped probability distributions of gene frequencies are to be expected.

Using equation (1) we have performed Monte Carlo simulation for more than two alleles at a locus. For tri-allele systems,

where the gene frequencies of the population can be represented as the three distances from the sides to an interior point in an equilateral triangle, the probability distribution for the equilibrium surface can be plotted as a surface above the triangular domain. Values of b that give U-shaped gene frequency distributions for two-allele cases give three-allele gene frequency distributions that have modes in the three corners and very low probabilities in the centre of the triangular domain. The same is true for four or more alleles.

The foregoing results apply to infinite populations. The situation for finite populations, where some sampling error (drift) is unavoidable, can be sketched. Robertson¹ introduced the idea of a retardation factor, which shows the strength of an overdominant polymorphism for slowing fixation. This depends on both the size of a population and on the deterministic frequency of equilibrium. For population sizes greater than 1,000, equilibrium frequencies in the range 0.2–0.8 are quite secure—the mean time to fixation is very long. When the equilibrium frequency is near 0 or 1, however, the rate of fixation may be even faster than for a purely neutral case.

By tending to fix those equilibrium frequencies in the ranges 0.05–0.20 and 0.80–0.95, such drift would change the A-shaped distributions in Fig. 1B to W-shaped distributions. It would also tend to square off the corners of a U-shaped distribution, but it would be impossible statistically to distinguish the two cases. Therefore, if our arguments are correct, gene frequency data cannot be used to determine whether most electrophoretic polymorphisms are neutral or selected.

Yamazaki and Maruyama² concluded that gene frequency data, in the form of contribution to relative heterozygosity, was inconsistent with the overdominant model. But this conclusion was based on the assumption that all equilibrium frequencies of overdominant polymorphisms are equally probable (corresponding to the heavy horizontal line in Fig. 1B); whereas, following the reasoning outlined in this paper, we believe that this assumption is not reasonable and that their conclusion is unwarranted.

We have not explained the phenomenon of the U-shaped allele frequency distribution. We have merely shown that it may be consistent with a selectionist model.

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Triploid pseudogamous biotype of the leafhopper *Muellerianella fairmairei*

THE sibling leafhopper species *Muellerianella fairmairei* (Perris, 1857) and *M. brevipennis* (Boheman, 1847) (Homoptera, Delphacidae) are widely sympatric in Europe. Studies of the biological differences between the two species, and of their hybridisation has revealed a remarkably high proportion of females of *M. fairmairei* sampled in the field and a gradual disappearance of males in laboratory colonies. *M. brevipennis* had a normal sex ratio in field samples and when reared in the laboratory¹. Additional cytogenetic studies now demonstrate that in *M. fairmairei* a pseudogamous female triploid biotype coexists with the diploid bisexual form.

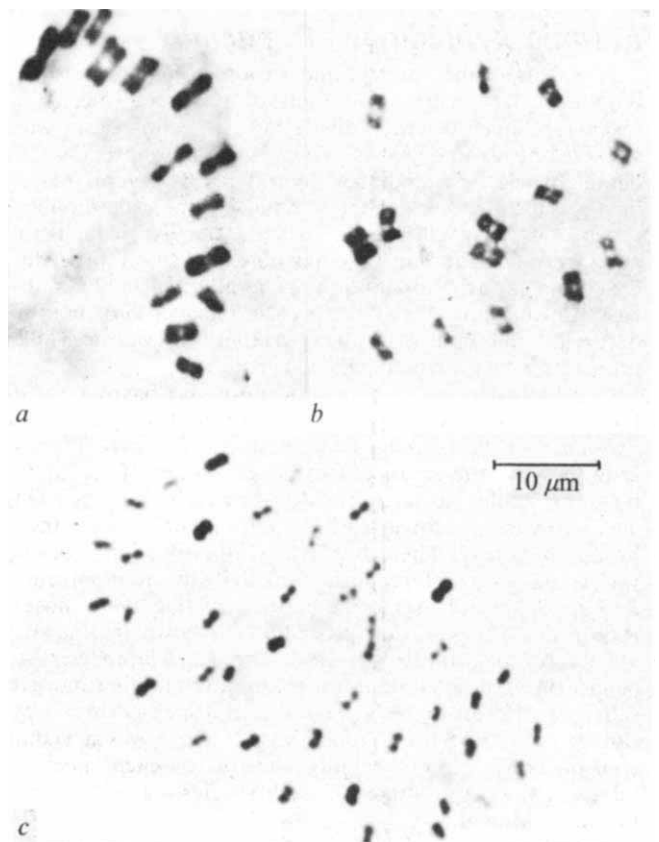
Populations of *M. fairmairei* (Southern Greece and Southern France) and *M. brevipennis* (Holland), which maintain a 1:1 sex ratio are diploid ($2n = 28$). In both species the male

karyotype consists of thirteen autosome pairs and one XY pair ($2n = 13 \text{ AII} + \text{XY}$). The female karyotype (Fig. 1a and b) shows thirteen autosome pairs and a large pair of XX chromosomes ($2n = 13 \text{ AII} + \text{XX}$).

Populations of *M. fairmairei* from Holland (Leersum) and England (Ascot-Silwood Park) with a high proportion (75–100%) of females seemed to comprise diploid males and a mixture of diploid and triploid females. Somatic metaphases of the triploid females, as far as could be determined, consistently had 41 chromosomes, including three X chromosomes. Ovarian semi-mature and mature eggs always contained a metaphase plate with 41 “pseudobivalents” (Fig. 1c). Their size was approximately half that of the bivalents of the diploid female, and they resembled secondary oocyte chromosomes. In this insect the first and second meiotic divisions probably do not take place and are replaced by a single equational division.

After the triploid biotype of *M. fairmairei* had been isolated in the laboratory, three experimental crosses were made to investigate the role of the sperm in the production of triploid females. When the cross was *M. fairmairei* ($3n$) ♀♀ × *M. fairmairei* ♂♂, five pairs reared on their host plant, *Holcus lanatus* (L.) produced triploid females only and in high numbers (160–514) females per pair. All five females appeared to have mobile sperm in their spermathecae. When the cross was *M. fairmairei* ($3n$) ♀♀ × *M. brevipennis* ♂♂, six pairs placed in a cage containing *H. lanatus* and *Deschampsia caespitosa* (L.) (the latter is the specific food plant of *M. brevipennis*) produced comparatively few triploid females (a total of 88). Three of the six crossed females had mobile sperm in their spermathecae. When the cross was *M. fairmairei* ($3n$) ♀♀ × sterile hybrid ♂♂, nine sterile males (produced by previous crossing of *M. brevipennis* ($2n$) ♀♀ × *M. fairmairei* ♂♂) were paired with females in glass tubes containing *H. lanatus*. Although the triploid females

Fig. 1 The chromosome complements of *M. fairmairei* ($2n$): a, *M. brevipennis*; b, *M. fairmairei* ($3n$); c, at the first female meiotic division, which occurs in the nucleus of semi-mature eggs. The bar in (c) represents 10 μm . All photomicrographs are reproduced at the same magnification.



were observed several times copulating with the hybrid males, none of the 163 eggs deposited exhibited embryogenesis.

Thus the triploid biotype of *M. fairmairei*, which is morphologically indistinguishable from the diploid female, has a typically pseudogamous mode of reproduction. The entrance of sperm (derived from the males of *M. fairmairei* or the closely related *M. brevipennis*) is necessary for embryonic development to occur, but the sperm probably do not fuse with the egg nucleus. Copulation with sterile males is ineffective. So far this mode of reproduction has not been reported in Hemiptera², although other forms of parthenogenesis are well known in this order.

When both female biotypes of *M. fairmairei* were reared together in the presence of males, the diploid type disappeared after one or two generations. The higher fecundity of the pseudogamous biotype and the fact that it produces all-female progeny possibly explain this phenomenon. It is not known how both biotypes can coexist in the field. It is clear, however, that the triploid type requires the availability of a diploid population for its maintenance.

An indication that the triploid biotype might be of hybrid origin is its ability to oviposit and develop on *D. caespitosa*, the host plant of *M. brevipennis*. Diploid hybrids, originated from crosses of *M. fairmairei* (2n)♀ × *M. brevipennis* ♂♂ and the reciprocal cross, have the same ability. Also, the meiotic behaviour of some female diploid hybrids (28 "pseudobivalents") closely resembles that of the triploid.

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Somatic hybridisation of *Petunia hybrida* and *P. parodii*

INTRASPECIFIC and interspecific fusion of plant protoplasts is induced by agents^{1–4}. Protoplasts of several species can regenerate a cell wall and divide to form callus, from which whole plants can be grown⁵. To combine these processes and obtain hybrid cells and then hybrid plants several requirements must be met⁶. The parental protoplasts should be capable of being cultured, as should the hybrid cells, and plant regeneration must be possible. Selection procedures developed so far^{7–10} may not be generally applicable, and we have now developed a method which makes use of naturally occurring differences in the sensitivity of cultured plant protoplasts to growth media and drugs¹¹.

One half of our selection procedure was based on a difference in growth between leaf protoplasts of *Petunia hybrida* and *P. parodii*. Protoplasts of *P. parodii*¹² never grow beyond the small colony stage (about 50 cells) in a medium similar to that of Murashige and Skoog¹³ (M/S medium) while protoplasts of *P. hybrida* cultivar Comanche¹⁴ produced callus¹⁵. The other half of the selection procedure was based on a difference in sensitivity to actinomycin D, *P. hybrida* protoplasts being more sensitive than those of *P. parodii*. The selection procedure is shown in Fig. 1.

Leaf protoplasts of *P. parodii* and *P. hybrida* were suspended in 9% (w/v) mannitol solution containing inorganic salts¹⁷ (2×10^5 protoplasts per ml) and dispensed into screw-capped tubes in 8-ml volumes so that there was a viability control, and a fusion viability control for each species. A mixture of equal volumes of each species was used for the fusion treatment.

All tubes, except viability controls, were centrifuged (80g,

10 min) and the supernatant was removed. To each tube was added 2 ml of 15% (w/v) polyethylene glycol (Koch-Light, molecular weight 6,000), 4% (w/v) sucrose and 0.01 M CaCl₂ to induce fusion. Protoplasts were resuspended, left for 10 min at 25 °C and then gradually diluted by the addition, at 5-min intervals, of the M/S medium in volumes of 0.5 ml, 1.0 ml, 2.0 ml, 2.0 ml, 3.0 ml and then 4.0 ml. Protoplasts were resuspended after each addition. After centrifugation at 60g for 15 min, the supernatant was replaced by M/S medium (8 ml per tube). At this stage the two viability controls were centrifuged and the protoplasts were resuspended in M/S medium. A further control was prepared by mixing equal volumes of *P. parodii* and *P. hybrida* fusion viability controls (post-fusion mixture control). All tubes were left for 1 h before plating. On average 4% of the protoplast population had fused as determined by counts of nuclei.

Protoplasts were maintained in liquid medium by the liquid-on-agar culture method¹⁸. To each 9-cm plastic Petri dish was added 8 ml of M/S medium containing actinomycin D ($1.0 \mu\text{g ml}^{-1}$), solidified with 0.5% (w/v) agar. To the surface of the agar was added 4 ml of M/S medium, containing actinomycin D ($2.0 \mu\text{g ml}^{-1}$), plus 4 ml of the appropriate protoplast suspension (at 2.0×10^5 protoplasts per ml), in M/S medium without actinomycin D so that the final concentration of the drug in the liquid layer was $1.0 \mu\text{g ml}^{-1}$, with a protoplast density of 1×10^5 per ml. Protoplasts of the two viability controls were plated in M/S medium without actinomycin D, as were samples of the *P. parodii* and *P. hybrida* fusion controls. Dishes were maintained at 27 °C with continuous illumination of 1,000 lx from daylight fluorescent tubes.

After 28 d, colonies were transferred to dishes containing M/S medium with 3% (w/v) mannitol solidified with 1% (w/v) agar and no actinomycin D. Growing colonies were transferred finally after 60 d to M/S medium containing no mannitol. Colonies were also recovered from the *P. hybrida* fusion control, maintained in the absence of actinomycin D, and from the *P. parodii*/*P. hybrida* fusion treatment in the presence of actinomycin D. All other dishes were devoid of colonies except the *P. hybrida* viability control. After a further 10 weeks, the small calluses of the *P. parodii*/*P. hybrida* fusion treatment, and the *P. hybrida* fusion control, were transferred to M/S medium, containing IAA ($2.0 \mu\text{g ml}^{-1}$) and 6-benzylaminopurine ($1.0 \mu\text{g ml}^{-1}$), for plant regeneration. Ten calluses of separate origin were recovered from the *P. parodii*/*P. hybrida* fusion treatment. Of these, eight produced shoots on this medium. Subsequently they were removed and rooted in M/S medium containing NAA ($0.1 \mu\text{g ml}^{-1}$) as the sole growth regulator and 0.3% agar. Plantlets thus produced were potted and grown up to flowering. Callus from the *P. hybrida* fusion control did not regenerate shoots on the same medium, although some shoots were produced when zeatin ($1.0 \mu\text{g ml}^{-1}$) was added.

No plants of *P. parodii* were recovered since the protoplasts did not grow beyond the colony stage. Plants produced from the *P. hybrida* fusion control were all diploid ($2n=14$), red flowered and like the parent species. All plants produced from *P. parodii*/*P. hybrida* fusion were purple flowered, and thus distinguishable from the parents (Fig. 2). Chromosome counts on roots showed a minimum number of 24, and a maximum of 28 in these plants.

Seedlings of *P. parodii* and *P. hybrida* were treated with colchicine (0.3% (w/v) aqueous solution) for 90 min to produce tetraploid ($4n=28$) plants. The F_1 sexual hybrid (*P. hybrida* × *P. parodii*) was obtained at the tetraploid level using colchicine. The reciprocal cross (*P. parodii* × *P. hybrida*) was identical in all respects. As Fig. 2 shows, flowers of the plants with 28 chromosomes, produced by fusion, were very similar to those of the tetraploid F_1 sexual hybrid. Tetraploid *P. parodii* and *P. hybrida* parents were

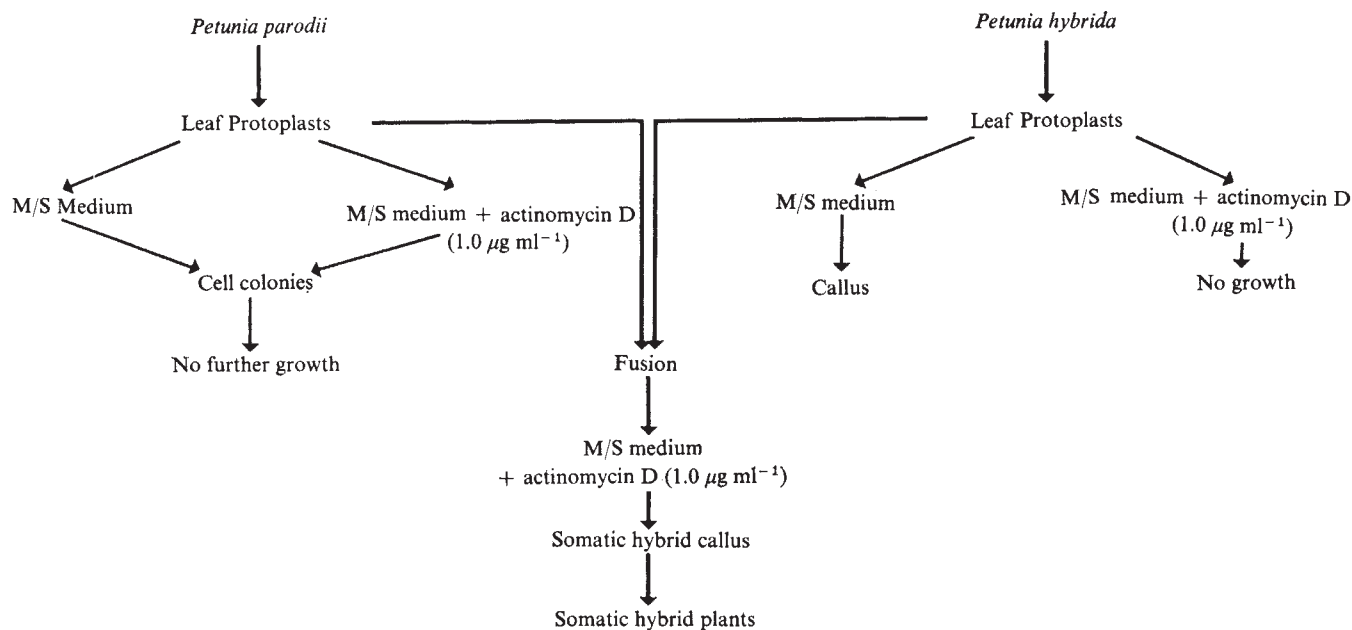


Fig. 1 Selection scheme for the production of somatic hybrids of *P. parodii* and *P. hybrida* based on differential parental protoplast growth responses to M/S medium and actinomycin D. Both species gave high yields of protoplasts ($2-3 \times 10^6$ per g fresh weight) with plating efficiencies of up to 40% in M/S medium, with naphthalene acetic acid (NAA) ($2.0 \mu\text{g ml}^{-1}$), 6-benzylaminopurine (6-BAP) ($0.5 \mu\text{g ml}^{-1}$), sucrose (3%) (w/v) and mannitol (9%) (w/v), pH 5.8. Division of *P. hybrida* protoplasts was limited by actinomycin D at $0.75-1.0 \mu\text{g ml}^{-1}$ in M/S medium, yet protoplasts of *P. parodii* were not affected up to $5.0 \mu\text{g ml}^{-1}$. In animal cells, actinomycin D resistance is dominant¹⁶, and likely also to be so in *Petunia*. Callus of *P. parodii*¹² readily undergoes shoot formation, yet that of *P. hybrida* does not¹⁵. It was expected that the regeneration potential of somatic hybrids would be provided by the *P. parodii* genome. In M/S medium plus actinomycin D protoplasts of *P. hybrida* would be expected to remain alive, but not divide while protoplasts of *P. parodii* would divide to the small colony stage. If complementation occurs in the somatic hybrid, the *P. hybrida* genome would permit division beyond the colony stage, while the *P. parodii* genome would confer resistance to actinomycin D. Protoplasts did not cross feed with respect to the M/S medium and actinomycin D growth responses.

easily distinguished from the F_1 sexual hybrid and the regenerated plants, for doubling of the chromosomes in the parents affected neither flower colour nor morphology. Therefore, the plants resulting from fusion that had 28 chromosomes were classed as somatic hybrids (*P. hybrida* + *P. parodii*). The sexual hybrid ($4n$) and the somatic hybrid (28 chromosomes) were both self-fertile and cross-fertile.

Determination of leaf isoperoxidase showed that the somatic hybrids had the spectrum of bands associated with both parents (diploid and tetraploid); and 20% of stained gels had an extra faint band found also in the tetraploid F_1 sexual hybrid (Fig. 3). The presence of this additional band, coupled with the hybrid nature of the flowers excludes the possibility that a chimaera had been obtained.

Fraction 1 proteins of *P. hybrida* and *P. parodii* were purified and analysed by A. A. Gatenby in our laboratory, using a specific immunoabsorbent, as described before¹⁸. Preliminary isoelectric focusing experiments of S-carboxymethylated protein, in the presence of 8 M urea, have revealed an identical polypeptide composition of the chloroplast-coded large subunit for both species. The small nucleus-coded subunit of *P. hybrida* contains two polypeptides¹⁹. *P. parodii* also contains two nucleus-coded polypeptides, both with isoelectric points identical to those found in *P. hybrida*. This is perhaps expected, for within the genus *Petunia* there seems to be a high degree of chromosome homology^{20,21}.

Experiments based on the same selection principle, and culture procedure, but with different concentrations of actinomycin D, have given selected calluses. Somatic hybrid plants have been produced after selection in M/S medium

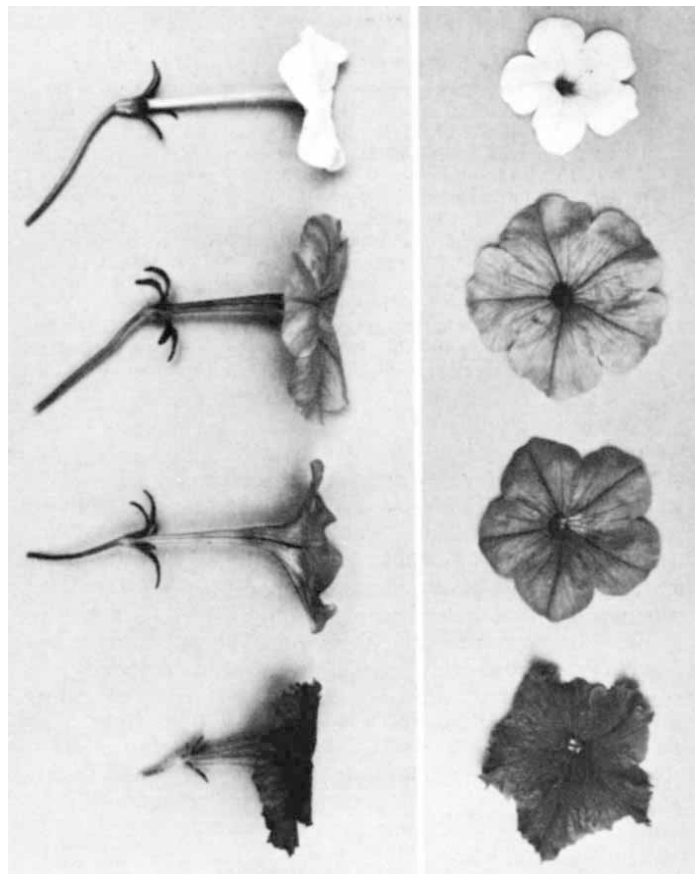


Fig. 2 Flowers of (from top to bottom) tetraploid *P. parodii* (white), tetraploid F_1 hybrid (*P. hybrida* $\times$ *P. parodii*) (purple), somatic hybrid (28 chromosomes) (purple) and tetraploid *P. hybrida* (red). The purple flower colour corresponds to *Fuchsia* purple (BCC No. 199, 28/1) and the red corresponds to Turkey red (Horticultural Colour Chart, 721). The somatic hybrid was also distinguishable from either of the parents on the basis of corolla tube and peduncle length. Petals of tetraploid *P. hybrida* were fringed, yet those of the F_1 hybrid and the somatic hybrid were smooth. Abaxial vein pigmentation of the floral bud was pronounced only in the F_1 hybrid and the somatic hybrid. Pollen of both parents was yellow, whereas that of the F_1 and somatic hybrids was occasionally purple.

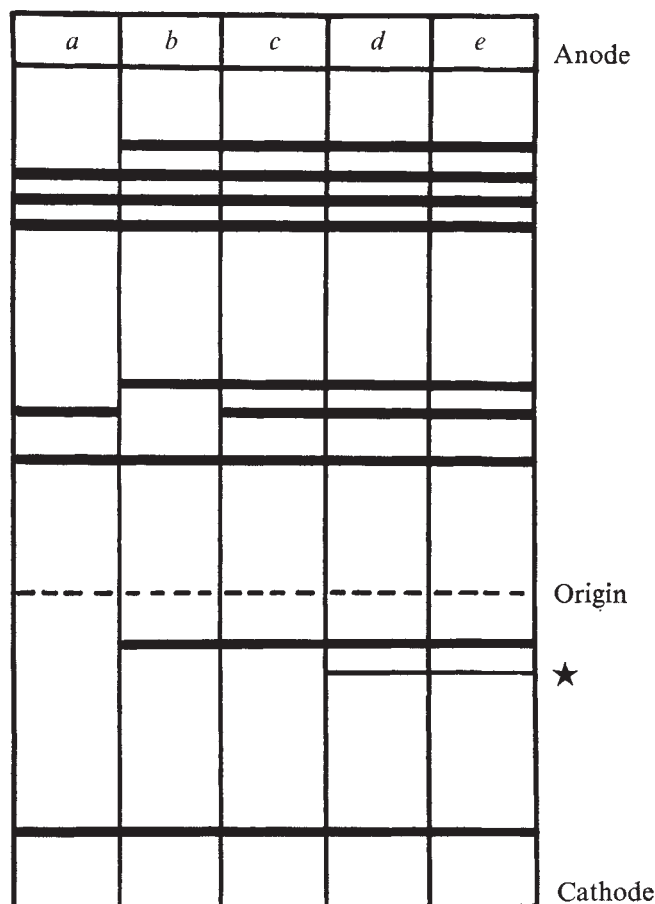


Fig. 3 Diagram of starch gel electrophoresis of peroxidase isoenzymes from leaf material of: a, *P. hybrida* (4n); b, *P. parodii* (4n); c, *P. hybrida* (4n) and *P. parodii* (4n) mixed before homogenisation; d, *P. hybrida* × *P. parodii* (4n); e, somatic hybrid (*P. hybrida* + *P. parodii*) (28 chromosomes). Leaf material was homogenised in 0.2 M sodium chloride solution at 4 °C using 1 g fresh weight of leaf per 0.5 ml of solution. A sample of the fresh homogenate was absorbed into a filter paper wick which was inserted into a freshly made 10% (w/v) starch horizontal slab gel (Electrostarch). The gel buffer contained 90% (v/v) 0.01 M citric acid and 0.065 M Trizma base, pH 8.2, plus 10% (v/v) 0.025 M lithium hydroxide and 0.2 M boric acid pH 7.9. The tank contained only the latter buffer. After electrophoresis for 6 h at 4 °C (40 mA), the gel was sliced horizontally, and a lower slice was stained for peroxidases using 100 mg of *O*-dianisidine in 70 ml of 95% (v/v) ethanol, plus 28 ml of acetate buffer and 2 ml of 3% (w/v) hydrogen peroxide solution. (The acetate buffer contained 0.14 M sodium acetate and 0.06 M acetic acid.) Staining was conducted at room temperature for 45–60 min, followed by washing in 70% (v/v) ethanol; photographed, and R_f values recorded. ★, Position of additional band.

with actinomycin D at 0.9 $\mu\text{g ml}^{-1}$, 0.8 $\mu\text{g ml}^{-1}$ and 0.75 $\mu\text{g ml}^{-1}$. At 0.75 $\mu\text{g ml}^{-1}$ the selection conditions allow some *P. hybrida* callus to grow through. In these circumstances, somatic hybrids were preferentially recovered since the shoot-inducing medium only rarely supports shoot formation in *P. hybrida*. So far, four separate experiments have produced somatic hybrid plants. Calluses have been selected in each of two further experiments. These calluses have regenerated shoots but have not yet been analysed fully. In one experiment which produced no somatic hybrids, actinomycin D (2.0 $\mu\text{g ml}^{-1}$) was added 10 d after fusion treatment, and *P. hybrida* was not sensitive to this concentration at the colony stage, and in the presence of *P. parodii*.

The frequency of somatic hybrid plant formation was variable but was of the order of one in $1-8 \times 10^5$ protoplasts. This does not take into account loss of protoplast viability

on plating or losses due to the effect of the fusion treatment.

Because neither mutant nor haploid plants are required, this selection strategy should be readily applicable to a wide range of species. We are investigating—using this complementation-selection procedure based on naturally occurring differences between species—the consequences of fusion of protoplasts isolated from sexually incompatible species.

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Pea root nodules containing more than one *Rhizobium* species

PARTICULAR species of legume are nodulated only by appropriate species of *Rhizobium*. Thus *R. leguminosarum*, *R. phaseoli* and *R. trifolii* nodulate peas, French beans and clover, respectively. The basis of this specificity between the two partners of the symbiosis is not understood. Complete specificity is not always found, however. Although *R. trifolii* does not normally nodulate peas, induced mutants of this species able to nodulate this host have been described¹ and some strains of *Rhizobium* isolated from clover nodules nodulated both clover and peas². Selected clover lines have been nodulated by *R. leguminosarum*³. It has been reported that a non-rhizobial contaminant can occur in soybean nodules⁴ and that the presence of *R. trifolii* may allow *R. meliloti* to occupy clover root nodules (B. O. Gillberg, personal communication). We show here that the presence of *R. leguminosarum* can allow *R. trifolii* and *R. phaseoli* to enter pea nodules but that *R. trifolii* cannot fix nitrogen in the nodules of this 'foreign' host.

The strains of *R. trifolii* (6001 and 6100) and *R. phaseoli* (1233) we used did not normally nodulate peas in our conditions. Of 15 plants inoculated with strain 1233 and 43 with strain 6001 none had any nodules, while one out of 36

plants inoculated with *R. trifolii* strain 6100 had three very small ineffective nodules. Since no viable bacteria could be isolated from them after surface sterilisation, strain 6100 could not be identified; conceivably they arose by contamination. The *R. trifolii* and *R. phaseoli* strains effectively nodulated clover and French bean plants, respectively.

The results of inoculating peas with various strains of *R. leguminosarum* together with *R. trifolii* or *R. phaseoli* are shown in Table 1. For each mixed inoculum at least one nodule on at least half of the plants contained the heterologous species. The frequency of such nodules was 2–27%. All nodules occupied by *R. trifolii* or *R. phaseoli* also contained the appropriate strain of *R. leguminosarum*. In most mixed nodules the heterologous species represented the minority of the bacteria recovered, but in some there were many more *R. trifolii* than *R. leguminosarum*. For example, from a nodule on a plant inoculated with strains 6100 plus 6099, 2.8×10^5 *R. trifolii* and 2.2×10^2 *R. leguminosarum* were recovered.

For all mixed inoculations of *R. trifolii* plus ineffective strains of *R. leguminosarum* there was no nitrogen fixation as determined by the absence of acetylene reduction whether *R. trifolii* was the majority or minority strain. Four of the *R. leguminosarum* strains (6009, 6099, 7111 and 7112) were derived from single mutagenic treatments from effective parents and thus can be assumed to be defective in one or very few steps. It is apparent that the defect(s) in these strains and of the two ineffective field isolates of *R. leguminosarum* (922 and 983) could not be overcome by the *R. trifolii* strains. Strain 922 does not inhibit acetylene reduction when it occurs in the same pea nodule as an effective strain of *R. leguminosarum*⁵.

A limited number of white clover (*Trifolium pratense*) plants was inoculated with *R. trifolii* strain 6001 plus *R. leguminosarum* strain 922 (which did not nodulate clover on its own). Of six nodules obtained from two plants, one contained both 922 and 6001. All six nodules were pink and effective, confirming that the presence of strain 922 in nodules did not inhibit nitrogen fixation by effective strains. This also demonstrates that occupation of nodules by heterologous species of *Rhizobium* is not restricted to peas.

Two independent *R. leguminosarum* strains (6007 and 6008) unable to nodulate peas were derived after ultraviolet

treatment of an effective strain (897). To determine whether the presence of non-infective strains of *R. leguminosarum* would enable *R. trifolii* to occupy pea nodules, peas were inoculated with *R. trifolii* (strain 6001) plus strains 6007 or 6008; no nodules were formed. Strains 6007 or 6008 do not inhibit nodulation by effective strains of *R. leguminosarum*. Neither of these strains was recovered from any of 237 nodules from plants inoculated with nodulating strains of *R. leguminosarum* plus strains 6007 or 6008. It is perhaps surprising that the presence of an infective strain of *R. leguminosarum* does not allow non-infective mutants of *R. leguminosarum* to enter pea nodules but does permit entry of strains of *R. trifolii* or *R. phaseoli*.

It should be noted that the techniques used here did not enable us to determine whether the heterologous species formed bacteroids in pea nodules since only the undifferentiated bacteria can form colonies on plates. Nor was it possible to determine where the heterologous strain occurred within the nodules. Transfer of the plasmid RP4 between strains of *R. leguminosarum* has been observed in pea nodules^{5,6}, but it was not found in any of the thirteen nodules that contained both *R. trifolii* and the RP4-carrying strain of *R. leguminosarum* (6099). RP4, however, is transferred by conjugation *in vitro* from strains of *R. leguminosarum* to strains 6001 and 6100 at much lower frequencies than it is to strains of *R. leguminosarum* (A.W.B.J., unpublished observations). The failure to find transfer in the nodules therefore need not imply that the two strains were physically separated.

It has been reported that the ability to nodulate clover can be transferred by conjugation from *R. trifolii* to *R. phaseoli*⁷ and by transformation from *R. trifolii* to *R. meliloti*⁸. In this study the results were not explicable by transfer of the ability to nodulate peas from *R. leguminosarum* to strain 6001 or 6100 since single colony isolates of these strains obtained from pea nodules retained their ability to nodulate clover but did not nodulate peas.

The bases of the specificity in the recognition between legumes and *Rhizobium* may reside in a barrier or barriers that are breached only by bacteria of the appropriate cross-inoculation group. By definition, infective strains of *R. leguminosarum* overcome these barriers in peas. A general explanation for the results presented is that the hetero-

Table 1 Incidence of pea nodules containing *R. phaseoli* (strain 1233) or *R. trifolii* (strains 6001 and 6100) in the presence of strains of *R. leguminosarum*

Heterologous strain	<i>R. leguminosarum</i> strain Stock no.	Genotype	Effective (E) or ineffective (I)	No. of plants examined	No. of plants with heterologous strain in one or more nodules	No. of nodules examined	No. of nodules with heterologous strain
1233	7482	<i>phe str-r</i>	E	2	1	22	3
6001	897	<i>phe trp str-r</i>	E	2	2	49	3
6001	6009	<i>phe trp str-r</i>	I	2	2	29	2
6001	7111	<i>phe</i>	I	2	1	22	1
6100	7111	<i>phe</i>	I	2	1	12	1
6001	7112	<i>phe</i>	I	1	1	12	2
6001	6099	<i>spc-r</i> (RP4)	I	1	1	43	2
6100	6099		I	5	5	58	11
6001	922	<i>his str-r</i>	I	2	1	45	1
6100	922		I	3	3	33	9
6001	983	<i>str-r</i>	I	2	1	30	1
6100	983		I	2	1	20	2

R. phaseoli strain 1233 is a rifampicin-resistant mutant of the pigment (probably melanin)-producing strain 3644 in the Rothamsted collection. *R. trifolii* strains 6001 (*rif-r*) and 6100 (*rif-r str-r*) were derived from strain CC37 supplied by E. A. Schwinghamer. *R. leguminosarum* strain 922 (*his rif-r*) was derived from ineffective strain 330 obtained from B. Gillberg. Strain 983 (*rif-r*) was derived from the ineffective Rothamsted strain 1012. All other *R. leguminosarum* strains were derived from our wild-type effective strain 300 (ref. 6): 6099-*(spc-r)* carries the plasmid RP4 and is ineffective; 603 (*phe*) was the parent of 897 (*phe trp str-r*) and 7482 (*phe str-r*); 6009 is an ineffective mutant obtained after ultraviolet treatment of 897; 7111 and 7112 were independent ineffective mutants obtained after nitrosoguanidine mutagenesis of strain 603. Culture and inoculation methods have been described before⁶. Peas (var. Dark Skin Perfection) were inoculated with about 2×10^8 of the inoculant strains. After about 4 weeks, nodules were excised, surface sterilised and crushed in 20% glycerol. Samples (0.04 ml) of the macerates were plated on suitable selective media^{6,9}. The macerates were stored frozen and those that contained the heterologous strain were diluted and plated on selective media to allow enumeration of the two strains.

logous *Rhizobium* species use the apparatus induced by *R. leguminosarum* so that there is a partial breakdown in specificity. The strains of *R. trifolii* and *R. phaseoli* used do not ordinarily induce nodules on peas but we do not know if they form infections which abort before nodule formation. *R. trifolii* was unable to fix nitrogen in pea nodules. This suggests that more than one barrier prevents *Rhizobium* of one cross-inoculation group forming an effective symbiosis with a legume of a different group. This is in accord with the findings that mutants of *R. trifolii* able to nodulate peas did not fix nitrogen in this host¹ and that some strains of *Rhizobium* isolated from clover formed ineffective nodules on peas².

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Erucic acid, an accidental additive in bread

RAPESEED oil is a stable, viscous oil utilised extensively by the baking industry in several countries as a lubricant to prevent the dough piece sticking to the container during baking. The oil is applied either to the container or directly on to the dough surface and allows the easy removal of the bread after baking. There are, however, few data available to indicate the extent to which any rapeseed oil is absorbed into the bread during processing. The question has assumed considerable importance since Roine¹ and many subsequent workers^{2–4} showed that a range of laboratory animals developed heart lesions and other biochemical abnormalities when fed diets with a substantial content of rapeseed oil. These effects have generally been attributed to erucic acid⁵ (13-docosenoic acid) which is the major fatty acid constituent in normal rapeseed oils (30–50% of total fatty acids)⁶. Consequently, various countries have legislated to restrict the amount of erucic acid permitted in certain foods, although no tolerances have been set specifically for baked products. This is mainly due to the assumption that erucic acid is not a natural component of the ingredients used in bread.

We have examined 20 different types of bread obtained from retail outlets in Sydney, Australia and found that all loaves contained erucic acid in concentration which ranged from 170 to 1,070 mg of erucic acid per kg of fresh loaf, with an average concentration of 405 mg kg⁻¹. A sample of wheat flour was also analysed but no erucic acid was detected (<0.2 mg kg⁻¹), and so the erucic acid in the breads can be assumed to be derived from the rapeseed oil applied just before baking. The breads analysed included plain and fancy rolls, and standard loaves of white, brown, whole-meal, rye, kibble wheat, soy flour–wheat and starch-reduced breads. The breads were dried and ground, and a sample (20 g) was extracted with solvent to remove the lipids which were saponified and methylated⁷, and analysed by flame ionisation gas chromatography using a 10% DEGS-PS on Supelcoport (80/100) glass column at 190 °C with a carrier gas flow rate of 20 ml per min of nitrogen.

Measurements were also made of the distribution of erucic acid throughout a single loaf. Table 1 shows that most of the erucic acid was present in the outer layer

Table 1 Distribution of erucic acid in a bread loaf (15 cm high)

Position in loaf	mg erucic acid per kg
Base–0.2 cm into loaf	9,550
0.2–1.0 cm	1,760
1.0–3.0 cm	50
3.0–7.5 cm (centre of loaf)	40

*Overall value for bread from base to 1.0 cm.

although some was found in the centre of the loaf. If these gradients can be applied to all loaves, then the crust slice (1 cm thick) would contain appreciable amounts of erucic acid and range from about 0.15 to 1.0% (w/w) of the crust.

Although much information has been obtained during the past 5 yr on the effects of erucic acid on animals, negligible data are available on its toxicological effects on man, and so it has been difficult to set meaningful levels of erucic acid permissible in human diets. An acceptable daily intake of rapeseed oil has been estimated to be 30 mg per kg of body weight⁸, which is equivalent to about 12 mg of erucic acid per kg. Our data show that an adult would achieve this intake if of the order of 1 kg of bread was eaten per day, although more than half the daily allowance would be supplied by two crust slices. Children, however, could receive more than their total allowable intake with only two crust slices. Thus, the use of rapeseed oil as a release agent in the baking industry leads to contamination of the product with erucic acid of a magnitude sufficient to cause concern for public health. The continued use of this substance on bread should therefore be reviewed. There are rapeseed oils readily available on the international market with low erucic acid contents (<1%)⁹ and these could be investigated for use in the baking industry. It is probable, however, that the viscosity of these oils would not be as desirable as that of high-erucic rapeseed oils, in which case, blending with other components would be necessary.

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Suppression of adenocarcinoma by the immunological consequences of calorie restriction

EARLIER extensive studies have indicated that calorie restriction as well as protein and amino acid restriction inhibit the spontaneous development of mammary or lung adenocarcinomas, hepatomas and certain chemical carcinogen-induced tumours in rodents^{1–4}. Jose and Good^{5–7} and Cooper *et al.*⁸ have shown that chronic moderate protein deprivation in mice and rats dramatically depresses antibody production while increasing, or permitting maintenance of, vigorous cell-mediated immune responses. The latter include abilities to resist virus infection, reject skin allografts and develop killer-cell activity

Table 1 Rate of appearance of mammary tumours (MT) and mortality in C3H female mice given diet with normal or restricted calories

Calories per day	No. of mice dead with tumours per total dead					Total dead MT/Total	%
	101-200	201-300	Days 301-400	401-500	501-600		
16	0	0	4/5	7/11	1/1	12/17	71
10	0/4	0	0/4	0/4	0/6	0/18	0

against syngeneic and allogeneic tumour cells. More profound chronic protein deprivation, however, depresses both cell-mediated and humoral immunity^{7,9}. Walford *et al.*^{10,11} have shown that calorie restriction delays development of immune functions at an early age but prolongs maintenance of immunological capacity and survival in long lived, tumour-free mice. Further, Fernandes *et al.*¹² have shown that dramatic prolongation of life of autoimmunity-prone, short lived mice was produced by life-long calorie restriction, and that tumours did not appear in these mice. Although some of the findings clearly link moderate protein and calorie restriction to heightened cellular immunity, no definitive efforts have yet been made to compare directly the influence of dietary restriction on spontaneous tumour development and immunological functions. This report presents observations on the influence of calorie restriction on development of spontaneous mammary adeno-

the development of spontaneous mammary adenocarcinoma in the female C3H/Umc mice, and more than 50% of them lived longer than 400 d. In contrast, 71% of the control group of female C3H/Umc mice on the normal (16 calories) diet developed mammary tumours by 500 d.

In Table 2 a comparison of mitogen response studies carried out by using the technique described previously¹³ is presented for the spleen cells obtained from mice on the two diets. Responsiveness of spleen cells to optimum concentrations of the T-cell lectins, phytohaemagglutinin (HA17) or concanavalin A (con A) was not only maintained on the low calorie intake but actually increased. The responses to the B-cell mitogen lipopolysaccharide (LPS) were also maintained at a level at least equivalent to that achieved by spleen cells of mice in the control group.

Table 3 summarises data comparing capacity to develop

Table 2 The incorporation of ³H-TdR with and without mitogenic stimulation of spleen cells from normal and calorie restricted C3H/Umc 3-month-old mice

Calories per day	Cultures* (h)	Controls	c.p.m. of ³ H-TdR (at optimum concentration of mitogens†)		
			+PHA (2.5 µg)	+con A (.5 µg)	+LPS (10 µg)
16	48	770±240	100,164±11,327	76,834±16,825	42,018±7,740
	72	467±170	74,369±4,597	101,274±20,959	20,051±4,669
10	48	1,636±194	144,018±11,087	103,752±10,354	43,122±734
	72	671±226	80,712±5,732	154,559±6,218	21,360±1,836

*0.5 × 10⁶ spleen cells from 4 individual mice per group cultured in RPMI-1640 media (triplicates) with foetal calf serum (2%) for 48 or 72 h at 37 °C in CO₂ and humidity in 3,040 microtest II tissue culture plates. Cultures were labelled with tritiated thymidine ³H-TdR (0.5 µCi per well) for an additional 16 h.

†Source of mitogens—PHA (HA17), Wellcome; con-A, Sigma; LPS, Difco.

carcinoma in mice and relates the suppression of tumour development observed to alterations in immune functions produced by calorie restriction at weaning.

Four-week-old female C3H/Umc mice were fed diets comprised of 22% casein, 33% dextrose, 33% starch, 5% corn oil, 4% salt mixture and 2% vitamin mixture, as reported previously^{5,8,13}. The protein-calorie-deprived mice were fed 10 calories instead of the 16 calories eaten by the control mice, but in all other respects the diets were the same. The food was provided in conditions in which each mouse consumed its entire ration each day. The influence of this form of chronic calorie restriction on survival of C3H/Umc mice is shown in Table 1. In mice of this strain, calorie restriction did not significantly alter lifespan, even though calorie-restricted mice lived longer (3/18) than any of those in the control group. Table 1 shows that the diet low in calories completely prevented

direct haemolytic plaque-forming cells (PFC) 4 d following (intraperitoneal) immunisation with sheep red blood cells (SRBCs)¹⁴. After the primary stimulation, the number of direct PFCs developed in the spleens of mice on a low calorie diet was markedly depressed as compared with that of the mice in the normal calorie group. In contrast, after a secondary stimulation, the PFC formation was not significantly different in the two groups. Of interest was the finding that when the spleen cells of both groups were compared for ability to generate a secondary PFC response after injection into lethally X-irradiated (950 R) recipients¹⁵, the sensitised cells from the calorie-deprived donors showed a greater number of PFC per million cells than was generated by spleen cells from sensitised mice which had been fed the higher calorie diet. Although several interpretations for this observation are possible, a likely explanation is that a suppressor cell population active in the

Table 3 Influence of normal and calorie restriction on immune response to SRBCs in C3H/Umc mice

Calories per day	Primary response* (direct PFCs)		Secondary response† (indirect PFCs)		PFC response in X-irradiated (950 R) host‡	
	10 ⁶	Spleen	10 ⁶	Spleen	10 ⁶	Spleen
16	127±17 (7)	10,131±1,123	231±121 (5)	19,625±9,223	95±22 (10)	2,058±749
10	40±15 (6)	2,091±984	181±13 (5)	13,327±2,409	190±34 (10)	1,668±404
Student's <i>t</i> test	<i>P</i> < 0.01	<i>P</i> < 0.01	NS	NS	<i>P</i> < 0.02	NS

*Immunised (intraperitoneal) with 10 × 10⁸ SRBCs for 4 d.

†Primed mice for 7 d with SRBC were again immunised and PFC assay was carried out on day 4.

‡Spleen cells (30 × 10⁶ or 40 × 10⁶) from immunised (primary) normal or low calorie mice were injected along with SRBCs into the lethally X-irradiated 8-week-old mice fed a normal laboratory diet. PFC assay was done on day 4 or 5 after injection.

Figures in parentheses represent the number of mice tested in each group.

Table 4 Antigen induced inhibition of DNA ($^{125}\text{IUdR}$)* synthesis in normal and calorie-restricted C3H 3-month-old mice

Calories per day	Body weight (g)	Injected with	Spleen weight (mg)	Per 100 mg spleen (c.p.m. $\pm$ s.e.)	% change
16	31 $\pm$ 2 (6)	Saline	54 $\pm$ 2	2,115 $\pm$ 13	
16	32 $\pm$ 2	SRBCs	79 $\pm$ 7	2,952 $\pm$ 406	+39
10	22 $\pm$ 1 (6)	Saline	48 $\pm$ 3	3,112 $\pm$ 36	
10	23 $\pm$ 1 (6)	SRBCs	68 $\pm$ 3	1,817 $\pm$ 174	-42

*2 μCi of $^{125}\text{IUdR}$ (Amersham/Searle, specific activity 106 $\mu\text{Ci } \mu\text{g}^{-1}$, Batch No. 294BA) was injected intraperitoneally 6 h after administration (intravenously) of saline or SRBCs. Twenty hours later mice were killed, spleens weighed and isotope incorporation was determined in a Gamma Counter (Nuclear Chicago).

calorie-deprived host is not as effective as it was in the intact animal at inhibiting immune response to SRBCs after transfer to the lethally X-irradiated syngeneic recipients. The irradiated mice given sensitised spleen cells from the calorie-deprived mice showed no increase, however, in absolute number of PFCs per spleen (Table 3). This might be attributed to differences in relative proportions of different subpopulations of donor cells and their capacity to migrate or to cooperate during the immune response in the spleen of X-irradiated host animals.

The technique of Zatz and Goldstein¹⁶, which may be one means to evaluate a population of suppressor T cells by their capacity to suppress DNA synthesis *in vivo* after injection of SRBCs, was then used to determine whether the suppressor T-cell influence is indeed excessive in the spleens of the calorie-deprived C3H/Umc mice. Table 4 shows that, by this analysis increased suppressor effect for this T-cell dependent response was also demonstrable in the calorie-deprived animals. It can be seen from Table 4 that, whereas no suppressor-cell influence was demonstrable by this technique in the spleens of 3-month-old mice fed the normal calories, those fed the low calorie diet showed very marked evidence of T-cell suppressor activity.

Our findings confirm those of several investigators¹⁻⁴ in showing that calorie restriction *per se* prevents development of mammary adenocarcinoma in highly susceptible C3H/Umc mice. They also show that moderate protein and calorie restriction permits maintenance of a vigorous functional thymic cell population, as has been found with chronic protein deprivation^{5,6,8,13}. Calorie restriction apparently also decreases the PFC response to T-dependent antigen in 3-month-old mice. That such a marked deficiency of PFC response to SRBCs is not due to the changes in the functional capacity of macrophages is suggested by data from a previous study⁸ which indicated that macrophage function is normal in protein-calorie deprived mice. Perhaps this issue still needs to be studied more extensively. The basis of the humoral immunodeficiency in the primary response of the calorie-deprived mice might be associated *in vivo* with an endocrine hypofunction¹⁷ which may permit a T-cell suppressive influence in the host. This interpretation is suggested by the observation that spleen-cell response *in vitro* to both PHA, con A and LPS was not altered by calorie deprivation, and that secondary PFCs in response to SRBCs are produced quite well when the previously stimulated spleen cells of the calorie-deprived animals are transferred into lethally irradiated hosts. This postulated relationship is compatible with the recent evidence presented with "T-dependent" and "T-independent" antigens, suggesting that a T-cell population present either in adult or newborn spleens possesses the ability to recognise feedback signals, and makes a decision to suppress or enhance the immune response on the basis of the amounts and molecular form of antigen given either in a suppressive or non-suppressive environment^{18,19}. More direct evidence for an active suppressor-cell population in the calorie-deprived mice, as has been revealed in young NZB mice²⁰, was then obtained by an assay which measures DNA synthesis in the spleen. The association of this immunological perturbation with resistance

to mammary tumours and with the marked change in hormonal functions²¹ and their influence on target tissues or receptor sites of a host given a calorie-restricted diet deserves extensive study.

An interesting possibility suggested but not proved by these findings is that T-suppressor cells might suppress directly the development of mammary cancer. This is a remote possibility but it should be studied directly. Another possibility is that the calorie-deprived mice whose lymphoid system is so much perturbed might lack the ability to develop still another form of suppressor cells. This latter cell type has recently been shown to occur in animals during tumour growth, and is implicated in the inhibition of cellular immune functions²²⁻²⁵. The elimination of the development of such suppressor-cell activity and/or absence of serum-blocking factors^{26,6} by calorie restriction might facilitate the destruction of newly arising tumour cells by immunocompetent T lymphocytes and macrophages. The nature and mode of such immunological alterations in relationship to dietary restriction remain unknown, and further studies are urgently required to elucidate the role of nutritional manipulations in susceptibility and resistance to tumour development *in vivo*. Similarly, although these studies and previous investigations from our laboratories seem to suggest that immunity could be involved in the resistance to tumours produced by low calorie diet, much further study will be needed to elucidate these relationships.

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Mechanisms of bone destruction in the development of skeletal metastases

SKELETAL metastases are associated with progressive bone destruction but the pathogenesis is not well understood. I report here experiments which indicate that two principal mechanisms are involved. The first is mediated by osteoclasts and the tumour secretes a diffusible osteoclast activating factor; the second functions when the residual trabeculae are surrounded by the tumour cells.

Several hundred specimens of bone, obtained at autopsy from the spines of 68 patients who died from various malignant diseases¹, were fixed in neutral formalin and decalcified in 5% nitric acid until no residual calcification was seen radiographically. Sections were then stained with haematoxylin and eosin and examined microscopically.

A suspension of VX2 carcinoma was obtained by finely dividing approximately 1 g of tumour in 5 ml of 0.15 M NaCl, producing approximately 2.5×10^6 cells per ml. In the first group a 0.5-ml sample of the suspension was injected into one tibia or ilium of New Zealand white rabbits or on to the periosteal surface. With tibial implants (16 rabbits), the bone was exposed at operation under ether anaesthesia, and a fine drill hole, just large enough to accommodate a size 18 needle, was made through one cortex. The tumour cell suspension was injected well down the medullary cavity using a bent size 18 needle. In the first few experiments the hole was plugged with bonewax, but this was found to be unnecessary and was discontinued. Iliac implantation was made by percutaneous injection into the iliac crest (six rabbits) or into a hole drilled in the ilium at open operation (six animals); all the periosteal injections were percutaneous (12 rabbits).

In half the animals 0.15 M NaCl was injected into the contralateral bone; in the others no operation was performed on this bone. The rabbits were killed at intervals from 24 h to 8 weeks later, and specimens from both the injected and the contralateral bone were treated in the same way as the human necropsy specimens. In the second group of 33 rabbits, 1 ml of tumour cell suspension was used by the other experimental details were the same.

Fig. 1 Metastasis from prostatic carcinoma. There is considerable new bone formation (B) in the stroma of the tumour. Multiple osteoclasts (arrowed) can be seen, adjacent to areas of bone destruction ($\times 96$).

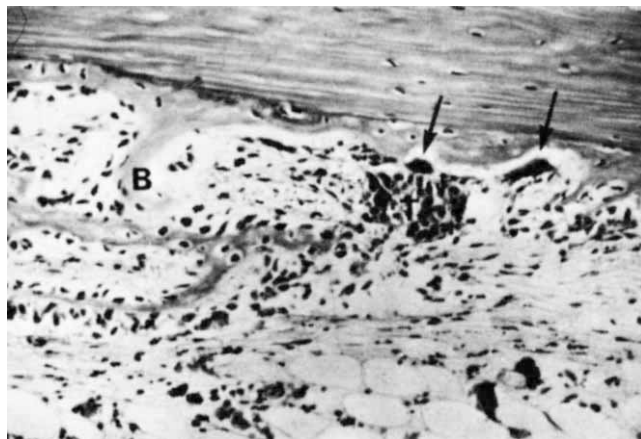
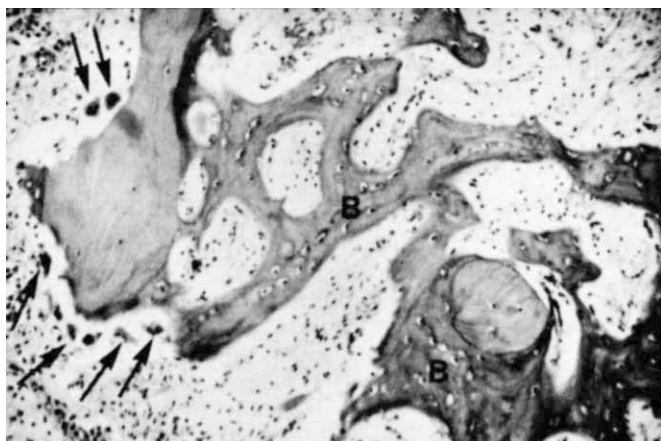


Fig. 2 Section taken 24 h after the injection of VX2 carcinoma. Osteoclasts are prominent (arrowed) near the tumour mass (T), and each is associated with localised bone destruction. Osteoblastic proliferation with new bone formation is also present (B) ($\times 120$).

In the third group (four rabbits) the tumour cell suspension was placed in a diffusion chamber with a 0.45- μ m Millipore filter, which was then implanted near the ilium but separated from it by normal muscle. In two of these rabbits a diffusion chamber containing saline was inserted on the contralateral side; the other two had no contralateral operation. The rabbits were killed 8 d later and sections from the left and right pelvis were treated as described previously.

In the fourth group, 4 ml of tumour cell suspension was injected percutaneously into the thigh muscle close to one femur, and the animals were killed 45–55 d later. Sections obtained from the distal and proximal tibiae and the femora were treated as described before. In addition, counts were made of the osteoclasts in each cross section, using a magnification of 200. At least two sections from each site were examined.

Study of the post-mortem sections suggested that there were two distinct mechanisms for the bone destruction. In many instances the bone surface was lined by numerous osteoclasts with local resorption occurring in the lacunae around each osteoclast (Fig. 1). In other sections the bone edge had a trabeculated appearance, suggesting that this was due to osteoclastic lacunae, although no osteoclasts were seen in the lacunae. This occurred with all types of tumour. Osteoclastic proliferation and bone destruction were independent of the associated osteoblastic reaction and new bone production¹. The osteoclasts were not part of the tumour mass, and usually fibrous reactive stroma filled the space between the edge of the tumour mass and the osteoclasts. Osteoclasts were seen when the associated bone destruction was not gross, but when there were only residual spicules of bone surrounded by tumour cells, osteoclasts were absent.

The first change seen in the rabbits (at 24 h) was osteoclastic proliferation separated from the tumour by a small amount of fibrous stroma (Fig. 2). Surrounding each osteoclast was a small lacuna with bone destruction. Some osteoblastic reaction with new bone formation was also seen at this stage. As the tumour grew, osteoclasts proliferated. As the tumour edge approached the endosteal surface of the cortex, localised destruction occurred in which osteoclasts were prominent. Once the tumour mass actually reached the endosteal surface of the residual cortex, osteoclasts were seen in large lacunae within the cortex. At this stage, there was periosteal new bone formation, associated with the osteoclastic proliferation and cortical bone destruction. When the tumour was injected on to the periosteal surface osteoclastic destruction of the



Fig. 3 Metastasis. Residual trabecula of bone (B) surrounded by tumour (T). Osteoclasts are absent ($\times 96$).

cortex, extending from the periosteum to the endosteal surface occurred, the osteoclasts always preceding the tumour.

The osteoclasts disappeared once the tumour had grown sufficiently large to envelop a residual trabecula of bone, but bone destruction continued (Fig. 3). In a few animals osteocytic osteolysis occurred in the tibial cortex, but this accounted for only a minute amount of the bone loss. Occasional infarcts were seen. They usually included bone as well as tumour, and were responsible for only a small amount of the total bone destruction.

Tumour cells implanted in the diffusion chamber remained there as shown by subsequent histological examination, as did the normal muscle between the chamber and the ilium. Osteoclastic proliferation was seen on the periosteal surface and even in the medullary cavity. There was no osteoclastic proliferation on the contralateral side.

Tremendous osteoclastic proliferation occurred in the femur when tumour cells were injected into an adjacent muscle. Their number diminished progressively as the distance from the tumour increased. There was no

osteoclastic proliferation on the contralateral side (Fig. 4).

These experiments suggest that destruction of bone associated with metastatic invasion involves at least two mechanisms, and possibly others of less importance. The initial mechanism, apparently the more important quantitatively, was mediated by osteoclasts. The diffusion chamber experiments suggest that the tumour secreted a humoral factor which stimulated osteoclastic proliferation locally but not on the contralateral side. This was confirmed by the experiments with large amounts of tumour cell suspension implanted in the thigh muscle; the number of osteoclasts progressively decreased distally. Since the contralateral side was unaffected, the results indicate that if this substance enters the circulation, it is rapidly inactivated. Further experiments suggest that prostaglandins are involved².

The tumour cells seem to mediate late bone destruction by a mechanism not involving osteoclasts. Osteocytic osteolysis seems of little importance; it was found in the tibial cortex in only a few rabbits and caused only a minute amount of total bone destruction. It was not seen in the human necropsy material. Tumour proliferation may be associated with thrombosis of the vessels and ischaemia, but this was not a feature in the experimental animal or in the necropsy material.

Previous studies have shown that skeletal metastases are associated with both new bone formation and destruction, which occur simultaneously¹. There seem to be at least two mechanisms for the bone formation. When the tumour provides a suitable matrix for ossification, bone is laid down if osteoprogenitor cells are available, for example, metastases from prostatic carcinoma. New bone is formed in all metastases once there is some bone destruction. This is similar to callus repair after a fracture and may be due to stress on the weakened bone¹. The present studies suggest that this occurs during osteoclast-mediated bone destruction. Once the destruction was gross and malignant cells surrounded the residual bone, no new bone was formed and no osteoclasts were seen. This may explain the occasional false negatives in skeletal scintigrams when X-rays demonstrate large lytic metastases—at this stage there is no reactive new bone to concentrate the bone-seeking isotope.

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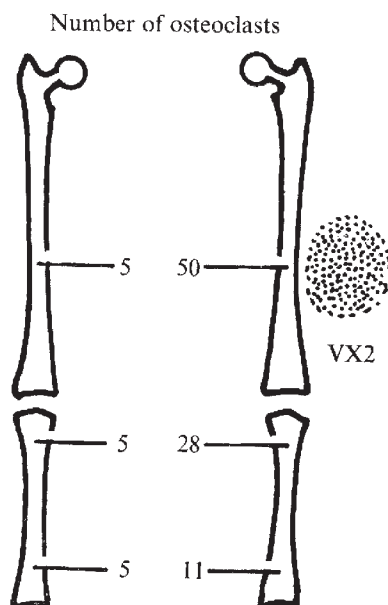
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Fig. 4 VX2 cell suspension injected into the left thigh, and the number of osteoclasts per cross section of bone counted at various sites. The number progressively diminishes the more distal the section from the tumour. There is no osteoclastic proliferation on the contralateral side.



Relationship of bone destruction in skeletal metastases to osteoclast activation and prostaglandins

Two principal mechanisms seem to be responsible for the bone destruction associated with skeletal metastases. The earlier and quantitatively most important mechanism seems to be mediated by osteoclasts stimulated by material produced by the tumour¹. We now report evidence that this material is, or contains, prostaglandin. There are precedents for such a conclusion. Various tumours contain prostaglandins², which resorb bone in organ culture³. Prosta-

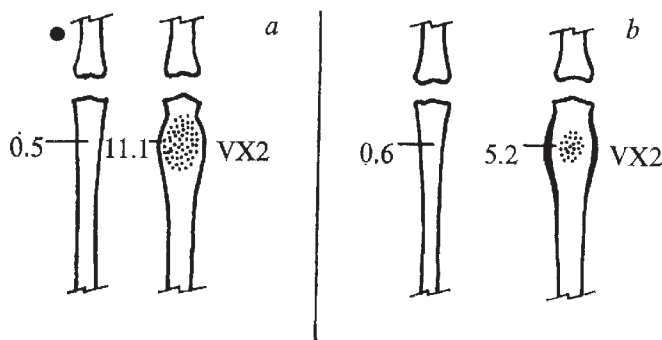


Fig. 1 The effect of indomethacin on osteoclast proliferation near the VX2 carcinoma. There is a marked reduction in the number of osteoclasts (per 100 possible sites) in the tumour-bearing tibiae but no change in the control tibiae. *a*, Control; *b*, treated with indomethacin.

glandin formation may explain the growth of dental cysts in the human jaw⁴, and prostaglandin synthetase inhibitors reduce tumour-induced osteolysis⁵ and hypercalcaemia⁶.

The experimental model used is good for investigating skeletal metastases. Approximately 1 g (fresh weight) of VX2 tumour, grown in the thigh muscles of donor New Zealand white rabbits, was finely divided by scissors in 5 ml of 0.15 M sterile NaCl, producing a suspension containing approximately 2.5×10^6 cells per ml. In 16 New Zealand white rabbits 1 ml of cell suspension was injected into the thigh muscle. Eight animals were given indomethacin 7.5 mg per kg body weight per d in their drinking water, starting at intervals varying from 7 d before tumour implantation to immediately after implantation.

The other eight animals, paired with animals receiving indomethacin, served as controls. Each pair received tumour cells from the same donor and was killed simultaneously. Tumours were examined for prostaglandins by homogenising weighed amounts in either Krebs' solution (to indicate synthesising ability) or in 50% ethanol acidified to approximately pH 3 with formic acid (to indicate "basal" levels)⁸. The prostaglandin-like material was extracted and assayed on the rat fundus strip preparation against prostaglandin E₂ (PGE₂)⁹. Part of the extracted Krebs' solution homogenate was characterised by AII solvent system chromatography using paper impregnated with silica gel¹⁰ followed by bioassay.

In 16 other rabbits 1 ml of tumour cell suspension was

Fig. 2 X rays of tibiae removed 31 d after injection of VX2 tumour cell suspension. The tibia on the left is from the control animal, that on the right from the indomethacin-treated animal. There is a marked reduction in the amount of bone destruction in the indomethacin-treated animal.



injected into the proximal tibia through a fine drill hole which exactly fitted a bent 18-gauge needle. Half the animals were treated with indomethacin (7.5 mg per kg body weight per d in the drinking water starting 1–7 d before tumour implantation). Indomethacin-treated animals were paired with controls which received tumour from the same donor. Radiographs of the tibiae were taken at intervals and when each pair was killed simultaneously 4–45 d later. Both tibiae were removed, fixed in formalin, decalcified in 5% nitric acid and embedded in wax. At least three histological sections, 1 mm apart, were cut from the proximal part of each tibia, and stained with haematoxylin and eosin. The osteoclasts were counted in each section using a cross-hatched eyepiece graticule and a magnification of 200: an osteoclast was counted if it lay under one of the cross hatches, but not if it lay between two cross hatches. Six hundred randomly selected sites were examined in each section and the number of osteoclasts per 100 sites was calculated.

The animals drank the indomethacin-treated water and the dose of indomethacin required was achieved. In both experimental groups, the lungs at autopsy were filled through the trachea with 15% Indian ink in distilled water containing 1 ml of ammonia per l of solution, removed and fixed in acetic acid–ethanol–formalin–distilled water (2 : 28 : 4 : 66 v/v). The pulmonary metastases, appearing as white areas on a black background, were counted on the external surface and in 1-mm slices through each lobe. The statistical tests included the paired *t* test.

High concentrations of prostaglandin-like material were extracted from the tumours from animals not receiving indomethacin (Krebs' solution homogenates 4.73 ± 1.06 (s.e.) μg PGE₂ equivalents per g; acid ethanol homogenates 2.4 ± 0.63 μg). The difference between these results is statistically significant ($P < 0.01$). Tumours from indomethacin-treated animals contained significantly less prostaglandin-like material (0.19 ± 0.07 and 0.24 ± 0.15 μg PGE₂ equivalents, Krebs' solution and acid ethanol homogenates respectively, both significantly less than controls, $P < 0.01$). Paper chromatography of extracted Krebs' solution and acid ethanol homogenates (nine and seven tumours, respectively) indicated that all the material was PGE₂. The activity ran fractionally slower than authentic PGE₂, but pure PGE₂ added to the extracts (three experiments) was slowed to the same extent presumably due to extracted impurities.

The number of osteoclasts in the tumour-bearing bone was significantly less in the indomethacin-treated animals (5.2 ± 1.71 osteoclasts per hundred sites) compared with controls (11.1 ± 2.13 ; $P < 0.05$) (Fig. 1). The numbers were low in the contralateral tumour-free tibiae of indomethacin-treated (0.6 ± 0.09 osteoclasts) and control animals (0.5 ± 0.22 osteoclasts). X rays revealed that in the indomethacin-treated animals bone destruction was markedly diminished but not prevented (Figs 2 and 3).

The number of pulmonary metastases tended to be higher (253 ± 67) in the indomethacin-treated animals than in the controls (189 ± 40), but the difference was not statistically significant ($P > 0.4$).

There is substantial evidence that secretion of prostaglandin by the tumour cells contributes to the activation of osteoclastic bone destruction. The tumour seems to be able to synthesise substantial amounts of PGE₂ as judged by chromatography and bioassay. This supports the conclusion of Voelkel *et al.* based on radioimmunoassay, the only difference being the amount of PGE₂-like material present. We found 4.73 ± 1.06 μg PGE₂ equivalents per g fresh tissue in our Krebs' solution homogenates. This represents the "basal" amount present plus prostaglandin newly synthesised during homogenisation, less any inactivated. Our "basal" levels are indicated by the acid ethanol homogenates where enzyme activity is inhibited.



Fig. 3 X rays of tibiae removed 45 d after injection of the VX2 cells. The tibia on the left is from the control animal, that on the right from the indomethacin-treated animal. There is a marked reduction in the amount of bone destruction in the latter.

The "basal" values of $2.4 \pm 0.63 \mu\text{g PGE}_2$ per g are surprisingly high compared with other tissues (for example, human breast^{11,12}). Voelkel *et al.* call their level of $0.294 \pm 0.051 \mu\text{g PGE}_2$ per g fresh weight "cortent", but since this was obtained after homogenising in a balanced salt solution, it presumably includes newly synthesised material and should be compared with aqueous homogenates. Apart from biological variation, their approximately 16 times lower levels might be due to losses during preparation and extraction (recoveries not stated); differences in time after implantation are unlikely to be important. Our studies confirm the findings of Voelkel *et al.* that feeding indomethacin to the rabbits greatly reduces the amount of extractable tumour prostaglandin. Although they looked at hypercalcaemia whereas we looked at bone destruction, both sets of data strengthen each other.

The reduction of the number of osteoclasts by indomethacin can be explained by the blockade of prostaglandin synthesis which this drug produces¹³. The failure to eliminate all production of osteoclasts and bone destruction might be due either to incomplete inhibition of prostaglandin synthesis, or to the involvement of other osteoclast-activating material (for example, a parathormone-like principle¹⁴). Furthermore, it is clear that bone destruction can occur without osteoclast involvement in late stages when the tumour cells surround the bone. It therefore seems likely that indomethacin or similar drugs would inhibit both the formation of human bone metastases, and their growth in the early stages. This supports the trial undertaken with benorylate in breast cancer patients with no evidence of bone metastases (personal communication from T. J. Powles), particularly as the synthesis of prostaglandin-like material by human breast tumours may be involved in the development of bone metastases^{11,12}. Indomethacin has been used in the treatment of hypercalcaemia due to malignant disease¹⁵ but was not successful in advanced mammary cancer (T. J. Powles, personal communication). These metastases have not been examined histologically, but their radiological findings are consistent with stage II disease¹.

In rats with Walker tumour, aspirin and indomethacin inhibited the hypercalcaemia and osteolysis, demonstrated radiographically, but not examined histologically². There was no significant difference in the soft tissue deposits; the tendency for more pulmonary metastases in our experiments was not statistically significant, and indomethacin treatment did not alter longevity (unpublished data). These results do not support the suggestion¹⁶ that PGE_2 is immunosuppressive and that indomethacin might retard tumour

growth. The formation of soft tissue metastases during treatment with indomethacin may have implications for treatment of bone metastases in human subjects, although it may not be appropriate to compare the effects of a massive injection of tumour cells into rabbits with a slower shedding of cells from human primary tumours. Presumably cells shed slowly from the primary tumour in the presence of indomethacin would not readily develop into skeletal metastases, but it remains to be seen whether they develop elsewhere. Chemotherapy would seem necessary to eradicate the tumour cells, and it might be desirable to treat the patients simultaneously with inhibitors of prostaglandin synthesis.

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Decreased renal prostaglandin catabolism precedes onset of hypertension in the developing spontaneously hypertensive rat

WE have shown in kidneys from normotensive rats, that dramatic age-related changes take place in the catabolism, but not in the biosynthesis, of prostaglandins (PGs)¹. Although PG catabolic enzymes were active in the late prenatal period, they rose to a peak (60-fold relative to the adult) around 19 d postnatally, dropping to adult levels by day 40 (ref. 1). We have termed this period of peak activity of PG catabolism the 'critical prostaglandin period'². Since the PG catabolising system is principally located in the cortex^{3,4}, and since cortical maturation is almost entirely a postnatal event (during the first 4–5 weeks) supported by an intrarenal blood flow redistribution⁵, the sharp increase in PG catabolism implied an intrinsic requirement by the developing kidney to eliminate locally-formed or plasma PGs. PG catabolism possibly functioned as a protective measure¹ against the potentially harmful effects of the PGs which in this species possess potent vasoconstrictor properties⁶. We theorised that unless inactivated, these compounds could interfere with the redistribution of blood flow to the cortex and consequently with normal corticogenesis. We were therefore interested in investigating the activity of the PG

system in spontaneously hypertensive rats (SHR) at various stages of pre- and early hypertension to compare this activity with age-paired groups of control normotensive rats in an attempt to determine first whether an abnormality in the prostaglandin system exists, second, whether this abnormality appears around the 'critical prostaglandin period' and, third, whether this abnormality develops before or after the onset of hypertension.

Litters (4–7 d) of male SHR of the Wistar Okamoto–Aoki strain and age-paired normotensive (NR) controls of the Wistar–Kyoto strain were purchased from Laboratory Supplies (Indianapolis) and housed in our animal quarters. Free access to food and water was provided. At various ages one rat from three SHR and three NR litters was killed. The kidneys from each group were combined and homogenised in twenty volumes of buffer (0.05 M KH_2PO_4 –NaOH, pH 7.4).

PG catabolism was assayed by the radioisotope-dilution method reported previously¹. Briefly, 0.5 ml homogenate (corresponding to 50 mg wet tissue, approximately 6.5 mg protein) was added to test tubes containing 5,6- $^3\text{H}_2$ -PGF_{1 α} (500,000 d.p.m., New England Nuclear, specific activity 89 Ci mmol⁻¹) containing 0, 1, 5, 10, 30 and 50 μg unlabelled PGF_{1 α} and NAD (4 mM final concentration) and tubes were incubated for 10 min at 30 °C. Ethanol (2.5 ml) was added to terminate the incubation and after centrifugation, the supernatant was transferred and evaporated to dryness *in vacuo*. Each sample was assayed qualitatively by radio thin-layer chromatography using chloroform–methanol–acetic acid–water (90:9:1:0.65 v/v) as developing solvent (R_f PGF_{2 α} = 0.13; 15K-PGF_{2 α} = 0.25, 15K-H₂-PGF_{2 α} = 0.36) and each product subsequently quantitated by scintillation counting as reported previously¹. Protein in homogenates was measured by the method of Lowry⁹.

PG biosynthetic capacity was measured by mass fragmentography using the stable-isotope dilution technique⁷. An aliquot (10 ml, corresponding to 0.5 g wet tissue, approximately 65 mg protein) of the same homogenate was incubated with excess arachidonic acid (100 $\mu\text{g g}^{-1}$) 10 min, 37 °C in an oxygen atmosphere. The incubation mixture was acidified to pH 3 with 0.5 N HCl and diethyl ether was added (10 volumes). A mixture of 3,3,4,4-tetradeuterated PGE₂, PGF_{2 α} and 15-keto-13,14-dihydro-PGF_{2 α} (1 μg each) was added followed by a mixture of high specific activity 5,6-tritiated PGE₁, PGF_{1 α} and 15-keto-13,14-dihydro-PGF_{1 α} (500,000 d.p.m. each, approximately 0.7 ng). After extraction, conversion to methyl esters and purification by thin-layer chromatography (R_f PGF_{2 α} Me = 0.28; PGE₂Me = 0.40; 15K-H₂-PGF_{2 α} Me = 0.48), each compound was quantitated as described previously⁸.

Table 1 shows the PG biosynthetic and catabolising capacity of

Table 1 Renal biosynthesis and catabolism of PGs by the normotensive (NR) and spontaneously hypertensive (SHR) male rat at various ages

Age		15-PGDH	PGE ₂ + PGF _{2α}
		ng per min per mg protein	ng per g tissue per 10 min
4 d	NR	76.5	
	SHR	100.1	
17 d	NR	221.6	380
	SHR	124.2	460
49 d	NR	8.9	1,620
	SHR	6.9	2,120
Adult	NR	7.6;6.5	1,520
	SHR	2.9;2.7	2,440

homogenates of kidneys from SHR and NR rats at four different ages. As observed previously¹ 15-PGDH catabolising activity varies immensely with age (about 60-fold around 3 weeks with respect to the adult) whereas the biosynthetic activity does not. Although the combined levels of PGE₂ and PGF_{2 α} at 17 d are approximately 20–30% of the adult, this difference can be largely explained by the high catabolising activity at 17 d. On the other hand, by comparing the relative capacities of 15-PGDH and the biosynthetic systems between NR and SHR rats it is evident that after 17 d of age the SHR rats possess lower renal catabolic activity. This is also supported by the apparent higher biosynthetic activity of SHR rats (Table 1).

The progressive difference between renal 15-PGDH activity in SHR and NR rats can best be seen in Fig. 1. Renal 15-PGDH activity in male adult SHR is approximately 40% of that found in NR. Before 1 week of age, SHR renal 15-PGDH activity is greater than NR decreasing to adult levels shortly after by 3–6 weeks. On the other hand, although no blood pressure differences are observed between the two types of rats at 3 weeks of age, differences become evident around 7 weeks of age. It is interesting that around this time a spurt in 15-PGDH activity occurs but this quickly returns to adult levels by 10 weeks of age.

The pattern of decreased 15-PGDH activity with age might not be typical of all types of hypertension. Armstrong *et al.*¹⁰ reported similar observations as ours with adult genetic hypertensive rats (GH) of the New Zealand strain but they failed to observe any differences with the Japanese spontaneous hypertensive rat. Unfortunately these authors did not carry out ontogenetic studies with either one of these strains, nor did they provide an indication of the types of PGs formed by these rats.

Our experiments demonstrate that (1) an abnormality in renal PG catabolism (Fig. 1) is evident in SH rats of the Wistar Okamoto–Aoki strain. (2) This abnormality develops in the pre-hypertensive stage reaching the adult level before the appearance of an elevation in blood pressure (3) higher PG biosynthetic capacity is observed in adult SHR than NR, but this reflects the lesser 15-PGDH activity. Thus it is possible that the hypertension that develops around 5–7 weeks of age in SH rats is due to renal 'damage' resulting from the inability to degrade effectively PGs especially during the 'critical prostaglandin period'.

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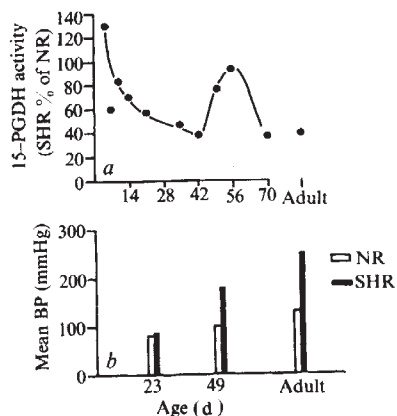
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Fig. 1 *a*, Age-dependent difference between the renal PG catabolising activity of spontaneously hypertensive rats (SHR) with respect to normotensive rats (NR); see text for experimental procedure. *b*, Mean arterial blood pressure measurements in SHR and NR rats at three ages. Values represent the mean of two experiments. Rats were anaesthetised with Inactin (100 mg kg⁻¹) and blood pressure was monitored by means of a Statham pressure transducer attached to a cannula in the carotid artery.



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Transformation of thymocytes by thymus epithelium derived from AKR mice

TYPE C RNA viruses result in the appearance of lymphomas and leukaemias when injected into genetically susceptible strains of mice¹. The relationship between the murine leukaemia viruses (MuLV) and their target cells in the induction of leukaemia remains, however, incompletely defined. Although MuLV viruses are ubiquitous in the tissues of many mouse strains and are present throughout the life of the animal, they transform specific haemopoietic cell types into leukaemic cells only after a relatively long latent period^{2–5}. This suggests that leukaemia is not caused by the virus alone but that a special interaction exists between viruses and target cells in a particular stage of differentiation and maturation.

The target cells of thymotropic MuLV such as radiation leukaemia virus and Gross virus seem to be thymocytes and/or prothymocytes, since the transformed cells have many T-lymphocyte characteristics and they first appear as neoplastic foci in the thymus^{6–8}. That differentiation events are important in leukaemogenesis is suggested by changes in the mitogen responsiveness of thymocytes during the latent period before the onset of disease and by change in the relative amounts of cell surface Thy 1 and H-2 antigens in both the spontaneous AKR- and radiation-induced C57BL mouse leukaemia models^{9–11}. In both of these systems the incidence of leukaemia is dramatically reduced after thymectomy, which demonstrates the central role played by the thymus^{12,13}. The thymic microenvironment may therefore be involved directly or indirectly, through factors released by the epithelium, in the induction of leukaemia by MuLV.

In earlier studies we have used cultures of pure thymus epithelial cells (TE) to simulate the thymic microenvironment *in vitro* to study the induction of differentiation of precursor cells into mature T lymphocytes^{14,15}. In the studies reported here, we used TE cultures to investigate the contribution of the thymic microenvironment to leukaemogenesis *in vitro*.

To analyse the role of target cell differentiation as a cofactor in leukaemogenesis we have used lymphocytes naturally in-

fectured with MuLV as targets for differentiation signals produced by TE. The AKR mouse provides a suitable model since MuLV (Gross type) has been detected in all cell types tested in this strain from before birth¹⁶. AKR mice develop leukaemia with 90% incidence only after they are 24 weeks old¹⁷. The AKR model would therefore allow us to study the role of differentiation and maturation of target lymphocytes while avoiding the need to add exogenous MuLV.

The experimental design was to culture TE cells from young non-leukaemic and aged AKR/J mice and to test the ability of these TE cells to transform naturally infected young AKR lymphocytes into neoplastic cells *in vitro*. The technique of TE cell culture has been published in full elsewhere¹⁸. Briefly, thymuses were removed aseptically, minced, and treated with trypsin-EDTA solution (GIBCO) and collagenase (Worthington Biochem). The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 15% foetal calf serum (FCS). Control monolayers consisted of kidney cells, from young and aged mice, as well as embryonic fibroblasts.

Thymocytes obtained from 6–8-week-old AKR/J mice were cultured together with supporting cell monolayers listed in Table 1 for 48 h. The thymocytes were collected from the culture dishes by gentle pipetting of the monolayer surface with Hanks' balanced salt solution (HBSS). In this way the monolayers are not disrupted and supporting cells are therefore not transferred along with the thymocytes. To ensure this, the culture dishes were each examined microscopically after the thymocytes were removed. The thymocytes were then washed in HBSS and resuspended in phosphate-buffered saline for injection into 8-week-old syngeneic recipients. Thymocytes cultured on TE monolayers from aged (more than 24 weeks old) AKR mice and then transferred into syngeneic recipients killed these animals after a short latent period. The animals died of lymphoma and leukaemia between 31 and 61 d after injection, as evidenced by splenomegaly, lymphadenopathy, and leukaemic nodules in the liver at post-mortem pathology. TE of aged AKR mice were effective in inducing leukaemia whether or not they were derived from the thymuses of overtly leukaemic mice. In contrast, thymocytes cultured on TE obtained from 4-week-old AKR mice did not kill the syngeneic recipients, and these animals died of spontaneous leukaemia only after 24 weeks of age as did uninjected control AKR mice. Also, as shown in Table 1, cultures of embryonic AKR fibroblasts or kidney epithelium from young or old, overtly leukaemic mice were unable to shorten the latent period.

These results indicated that the injection of the donor thymocytes led to the appearance of leukaemia in young syngeneic recipients. It was possible, however, that the leukaemia itself originated in the recipient mice and the leukaemogenic process was only hastened by the injection of the donor thymocytes. It was therefore necessary to ascertain whether true leukaemogenesis of the donor thymocytes had been induced *in vitro*.

We used AKR thymocytes with different alleles of the Thy 1 genetic marker to establish whether the recipient animals died of leukaemia or donor or host cell origin. AKR/J thymocytes, bearing the surface antigen Thy 1.1, that had been cultured on TE cells were injected into 8-week-old AKR/Cu mice whose T lymphocytes bear the Thy 1.2 alloantigen. In every AKR/Cu mouse studied the majority of the leukaemic cells in their spleens were positive for Thy 1.1, showing the disease to be of donor rather than host cell origin (Table 2). When AKR/Cu (Thy 1.2) thymocytes were cultured on TE for 48 h and injected into AKR/J (Thy 1.1) mice, the spleens of the recipients contained Thy 1.2 positive leukaemic cells (Table 2).

The genetic origin of the TE cells whether from AKR/J (Thy 1.1) or AKR/Cu (Thy 1.2) did not determine the *Thy 1* alleles of the leukaemic cells (Table 2). This argues against the possibility that the leukaemia was derived from the old TE cells themselves or from thymocytes that could have originated from the old AKR mice from which the TE cells were obtained. We concluded therefore that the leukaemia cells developing

Table 1 Survival of AKR/J mice receiving syngeneic 6-week-old AKR/J thymocytes cultured on thymus epithelium from aged mice

Inducing monolayers	Survival of 6-week old AKR/J recipients†
MEF (AKR/J)*	264 ± 63 d
Kidney epithelium (KE) (4 weeks)	251 ± 69 d
KE (> 24 weeks)	243 ± 74 d
Thymus epithelium (TE)	256 ± 70 d
TE (> 24 weeks)	46.8 ± 6.9 (31–61)

*Mouse embryonic fibroblasts.

†Survivals are reported as the mean ± s.d.

Thymocytes obtained from 6-week-old female AKR/J were cultured together with the inducing cell monolayers in 60 mm Falcon culture dishes (30 × 10⁶ thymocytes per 60-mm dish) containing RPMI 1640 + 15% FCS + 2-mercaptoethanol. After 48 h of culture the monolayers were washed by gentle pipetting with Hanks' balanced salt solution (HBSS). The thymocytes were washed in HBSS and resuspended in phosphate-buffered saline. Thymocytes from each group of inducing monolayers were injected intravenously into 8-week-old syngeneic recipients. 2 × 10⁶ thymocytes were transferred into each animal and each group consisted of 30 mice. The groups were then studied for survival. Only the group receiving cells cultured on TE cells derived from AKR mice over 24 weeks old died after a short latent period with a mean survival of 46.8 ± 6.9 d. The range was between 31 and 61 d. The animals in the control groups did not begin dying of leukaemia until after 150 d.

Table 2 Donor origin of transformed leukaemic cells *in vitro*

Donor thymocytes		Inducing TE cells from		Thy 1 type on leukaemic spleen cells		
Strain	Thy 1 Allele	Strain	Allele	Recipient	% Thy 1 $\pm$ s.e.	Thy 1.2
AKR/J	Thy 1.1	AKR/J	Thy 1.1	AKR/Cu	83 $\pm$ 1.2	8 $\pm$ 0.5
AKR/J	Thy 1.1	AKR/Cu	Thy 1.2	AKR/Cu	80 $\pm$ 0.9	6 $\pm$ 0.5
AKR/J	Thy 1.1	AKR/J	Thy 1.1	AKR/J	71 $\pm$ 1.6	5 $\pm$ 0.9
AKR/Cu	Thy 1.2	AKR/J	Thy 1.1	AKR/J	9 $\pm$ 0.8	79 $\pm$ 1.7
AKR/Cu	Thy 1.2	AKR/J	Thy 1.2	AKR/Cu	< 3	87 $\pm$ 1.4
AKR/Cu	Thy 1.2	AKR/Cu	Thy 1.2	AKR/Cu	< 3	78 $\pm$ 1.7

Thymocytes obtained from 6-week-old female AKR/J or AKR/Cu mice were cultured together with thymus epithelium from AKR/J or AKR/Cu mice more than 24 weeks old in RPMI 1640 + 15% foetal calf serum + 2-mercaptoethanol. Thymocytes were collected by gently pipetting the monolayers with Hanks' balanced salt solution. The thymocytes were resuspended in phosphate-buffered saline and 5×10^6 cells were transferred to 8-week-old AKR/J or AKR/Cu mice by intravenous injection. Each group contained 30 animals. Fifteen animals were killed after 30 d and cytotoxicity experiments were performed on individual spleens with anti-Thy 1.1 and anti-Thy 1.2 sera. Single-cell suspensions were prepared and the cell concentration was adjusted to 5×10^6 viable cells ml^{-1} with a final concentration of antisera at 1:8. Guinea pig complement was added after 30 min at a concentration of 1:4. Percentage viable cells was determined by Trypan blue exclusion. The results are reported as the means $\pm$ s.e. of the maximum cytotoxicity of individual spleens.

Also 15 animals from each group were studied for survival. 100% of the mice in each group died after a short latent period. The results show that in each group the leukaemic cells were of donor rather than host origin.

in the recipient mice originated from the donor thymocytes which had been incubated with the TE cells. Thus, the TE cells induced leukaemic transformation of young thymocytes naturally infected with MuLV.

Did the cultured thymocytes actually become leukaemic during the *in vitro* culture period? Did the TE cells provide a signal in itself sufficient for leukaemic transformation, or did they merely initiate a process that required further differentiation stimulated *in vivo* by the thymuses of the recipient mice?

To study these questions we performed thymectomy of recipient mice and injected them with donor thymocytes which had been cultured on TE cells. As shown in Table 3, we found that the donor thymocytes could manifest leukaemia even in thymectomised recipients. This finding suggests that the induction of leukaemia was initiated by contact with TE cells cultured *in vitro* and that continued contact with a thymus *in vivo* was unnecessary for leukaemic transformation. Thus 48 h or less of contact with TE cells *in vitro* was sufficient for triggering of leukaemogenesis.

In the present study thymus epithelium from aged AKR/J mice has been shown to initiate directly leukaemic transformation of thymocytes *in vitro*, in the absence of any added MuLV. When TE cells are injected into mice either subcutaneously or under the kidney capsule no tumour is formed. Injection of TE cells does, however, induce leukaemia in the recipient animals. Here the transformed cells are of host rather than donor origin. Mice receiving TE cells from old AKR mice die of lymphoma and leukaemia between 3 and 5 weeks (S. D. Waksal, in preparation).

What is the mechanism of this leukaemogenesis? Other tissues of aged AKR mice such as kidney epithelial cells contain replicating MuLV particles and yet were unable to transform thymocytes *in vitro*. In fact the target lymphocytes themselves already contain similar MuLV. Therefore, leukaemogenesis must involve more than the mere transfer of such viruses.

It has been shown that the thymus has an important role in this disease, since thymectomy up to 5 months of age can markedly inhibit the incidence of the disease in AKR mice¹². The thymus is responsible for the differentiation and maturation of thymocytes and thymus-derived lymphocytes. The target cell for both normal thymic activity and leukaemogenic transformation seems to be the prothymocyte or thymocyte^{8,19,20}. Thus the target cells of the leukaemogenic process might have to be in a critical stage of differentiation to be susceptible to the transforming potential of the MuLV with which they are already infected. The TE cells could activate leukaemia by inducing this necessary stage of differentiation. In support of this concept is our finding that mature lymph node lymphocytes cannot be transformed into leukaemic cells by old TE *in vitro* (S. D. Waksal, unpublished results). Such lymphocytes which have

already passed through the thymic microenvironment are also refractory to further differentiation caused by the TE cells *in vitro* (S. D. Waksal *et al.*, in preparation).

Leukaemic transformation of lymphocytes or their precursors has also been achieved *in vitro* using Abelson virus^{21,22}. Here too the state of target cell differentiation seems to be important since the target cells used for transformation are either lipopolysaccharide-stimulated spleen cells or foetal liver cells. The former population contains B lymphocytes non-specifically triggered to differentiate and the latter a population of cells which contain precursors differentiating into B lymphocytes.

It is, however, unlikely that a normal differentiation signal is the sole contribution to leukaemogenesis of the TE cells in our system. TE cells obtained from young AKR mice are capable of inducing the normal differentiation of thymocytes into T lymphocytes but do not provide the leukaemogenic signal (Table 1). Other factors must, therefore, distinguish old from young thymus epithelium. Such factors might include mechanisms which allow the endogenous MuLV to become leukaemogenic within the thymic microenvironment. There may also be a requirement for a second helper virus or viruses which replicate in the TE cells to produce leukaemogenesis in combination with the Gross-type MuLV. TE cells from aged AKR mice have significantly higher levels of replicating MuLV than TE cells from young mice. This has been observed using

Table 3 Effect of thymectomy on survival of AKR mice receiving syngeneic 6-week-old AKR/J thymocytes transformed by TE cells *in vitro**

Inducing monolayer (AKR/J)	Survival ATx recipient†
Kidney epithelium (4 weeks)	> 285 d
Kidney epithelium (> 24 weeks)	> 330 d
TE (4 weeks)	> 330 d
TE (> 24 weeks)	44 $\pm$ 6.9 d (32–53)

*Thymocytes from 6-week-old female AKR/J mice were cultured together with the inducing monolayers for 48 h. The thymocytes were collected by gentle pipetting of the monolayer surfaces and 5×10^6 cells were transferred into groups containing 25 syngeneic 8-week-old recipients. Each recipient had been thymectomised at 4 weeks of age. Only 1 out of 25 mice which had been adult thymectomised but received no transferred cells developed lymphoma with latent period of 187 days. AKR/J mice which had been thymectomised and received thymocytes cultured together with TE monolayers from AKR/J mice over 24 weeks old died of lymphoma after a short latent period (44 $\pm$ 6.9 d) with splenomegaly and lymphadenopathy. Only 2 animals in the control adult thymectomised groups died of leukaemia. The other control mice were still alive beyond days listed in Table 3.

†Survival of the last group is reported as the mean survival $\pm$ s.d. with a range of 32–53 d.

fluorescent anti-MuLV. These higher levels of virus may be critical in the development of leukaemia. These viruses might include the AKR xenotropic virus²³, which has been shown to appear only in TE cells derived from AKR mice older than 24 weeks.

The TE would then serve a dual role in the aged AKR; to trigger the cell into a susceptible state of differentiation and to supply the additional factors needed to initiate leukaemogenesis. Leukaemogenesis therefore seems to involve two or three distinct phases: (1) the infection with MuLV; (2) the necessary state of target-cell differentiation; and/or (3) additional unknown factors present in TE cells of aged AKR mice necessary to trigger leukaemic transformation. At present it is not possible to distinguish between the second and third possibilities. The long latent period needed for leukaemogenesis in the intact mouse might be required to generate such additional factors as the AKR xenotropic virus in the thymic microenvironment.

The *in vitro* system presented here makes feasible an analysis of the interactions of these factors in leukaemogenesis.

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Hormone-dependent haematopoiesis in fibroblast-transformation ossicles

SUBCUTANEOUS transplantation of coarse powders of acid-insoluble matrix of the shafts of long bones of rat to allogeneic recipients results consistently in the formation of new bone through endochondral ossification¹. Haematopoietic bone marrow develops in the newly formed ossicle in adolescent rats^{2,3} and this experimental model is amenable to an investigation of hormonal control of initiation of haematopoiesis. Erythropoiesis is dependent on pituitary hormones^{4,5} since hypophysectomy resulted in anaemia in all animal species which have been studied. Hypophysec-

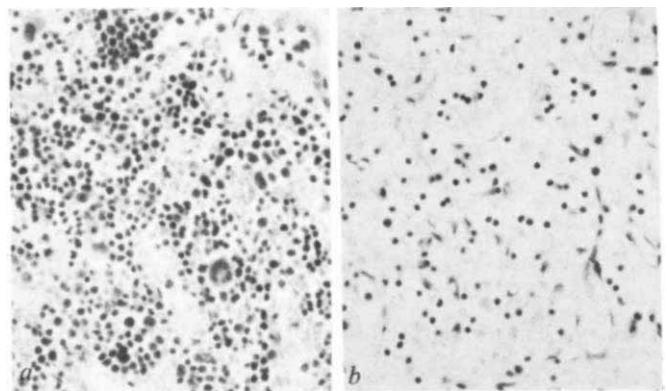


Fig. 1 Photomicrographs of histological sections of transformation ossicles from control (a) and hypophysectomized (b) rats stained with haematoxylin and eosin. a, Haematopoietic cells on day 26 in a control rat. b, Scattered basophilic cells in a hypophysectomized rat. Hypophysectomy on day 0 ($\times 250$).

tomy caused a prompt regression of many stem-cell erythroblastic leukaemias in rat⁶. We now report the profound influence of hypophysectomy on *de novo* erythropoiesis in matrix-induced transformation ossicles.

Dehydrated diaphyseal shafts of rat femur and tibia were comminuted and sieved. Powders with particle sizes of 74-420 μ m were demineralised with 0.5 M HCl and extracted with water, ethanol and ether as described in ref. 1. This preparation is designated as 'transformant of fibroblasts' (TF). Male rats, age 35-37 d, of Long-Evans strain were anaesthetised with ether. In sterile conditions TF (25-30 mg) was transplanted as a compact deposit into a subcutaneous pocket prepared by blunt dissection. The incision was closed with a metallic clip. As previous studies² have established the superiority of the thoracic site over that of the abdominal site for erythropoiesis, transplants were confined exclusively to the former anatomical location. In each rat there were two bilaterally symmetrical sites in the upper thoracic region. The day of operation was denoted day 0. Hypophysectomy was performed by D. Otway of the Hormone Assay Laboratory, Chicago, on day 0 unless otherwise indicated in the tables. At the time of collection of the transformation ossicles, the rats were anaesthetised and exsanguinated by cardiac puncture with a 19-gauge hypodermic needle followed by decapitation. The ossicles were dissected out and weighed. The weights of testis and spleen were recorded to confirm the completeness of hypophysectomy. The body weights of all the animals were recorded three times a week. The hypophysectomized animals lost weight whereas the control rats gained 3-4 g per day.

The histological sequences in response to subcutaneous implantations have been described in detail^{2,3} and consist of the following stages in the control rats. On day 1 a planoconvex, button-like plaque was formed; it is a conglomerate of TF, fibrin network and polymorphonuclear leukocytes. On day 3 the plaque consisted of TF and invading fibroblasts. By day 4 there was close-range matrix-membrane interactions³ and chondroblasts emerged on day 5 for the first time. Chondrocytes were abundant on days 7 and 8. With the incursion of capillaries on day 9 there began chondrolysis and osteogenesis. By day 12 an osseous conglomerate with cavernous spaces had formed. On day 14 extravascular islands of dark-staining haemocyto blasts were evident. With progressive differentiation the entire ossicle was filled with haematopoietic bone marrow replete with cells in the erythrocytic and granulocytic series and with megakaryocytes by days 23-25 (Fig.

Table 1 Influence of hypophysectomy on alkaline phosphatase and ^{59}Fe incorporation in transformation ossicles, femoral bone marrow and spleen

Day	Alkaline phosphatase U g^{-1}		Transformation ossicles		^{59}Fe incorporation (c.p.m. per mg tissue)		Spleen	
	Control	Hypox	Control	Hypox	Bone marrow Control	Hypox	Control	Hypox
10	60.0 $\pm$ 5	38.7 $\pm$ 3*	—	—	—	—	—	—
21	23.6 $\pm$ 2	60.3 $\pm$ 4*	77 $\pm$ 35	<1	1,671 $\pm$ 198	442 $\pm$ 120*	259 $\pm$ 47	28 $\pm$ 9*
25	29.8 $\pm$ 3	45.6 $\pm$ 2*	334 $\pm$ 49	1 $\pm$ 0.3	1,756 $\pm$ 147	971 $\pm$ 113*	486 $\pm$ 43	20 $\pm$ 3*
28	29.8 $\pm$ 2	41.7 $\pm$ 3*	579 $\pm$ 80	9 $\pm$ 0.4	2,541 $\pm$ 131	553 $\pm$ 95*	1,030 $\pm$ 138	30 $\pm$ 9*

Alkaline phosphatase and ^{59}Fe incorporation into protein-bound haem was determined as described earlier². All numbers represent mean $\pm$ s.e. of 8 observations.

*Significant difference from control.

1a). Collection was routinely carried out on days 27–31 in our experiments, as haematopoiesis became fully functional at that time.

Bone formation was assayed by measuring the activity in the transplantation ossicle of alkaline phosphatase, an enzyme known to be intimately associated with ossification⁷. Hypophysectomy (hypox) delayed but did not prevent the formation of the osseous conglomerate. The maximal concentration of alkaline phosphatase, $\sim 60 \text{ U g}^{-1}$, was similar (Table 1) in the ossicles of intact and hypox hosts. But the peak of enzyme activity in intact rats was attained on day 10 whereas in hypox rats, the concentration of alkaline phosphatase in the ossicles was a maximum (Table 1) on day 21.

Hypophysectomy profoundly inhibited the development of haematopoiesis. On day 27, the transplantation ossicles in normal hosts were dark red with an abundance of maturing blood cells (Fig. 1a). In contrast, the ossicles in hypox rats were pale pink in the gross on day 27 and the marrow consisted exclusively of scattered individual basophilic cells (Fig. 1b), presumably haemocytoblasts; maturation of blood cells was absent. The incorporation of ^{59}Fe into protein-bound haem, an indicator of erythropoiesis, was barely detected in transformation ossicles of hypox rats and was significantly lower than the controls in the femoral bone marrow (Table 1). The spleen of hypox rats was tiny and pale pink in the gross; the spleen of control rats was larger and had a dusky red colour (Table 2). The ^{59}Fe incorporation in the spleen was significantly depressed following hypophysectomy (Table 1).

In another series of experiments the day of hypophysectomy was varied to determine if a delay in removal of the pituitary would improve the ^{59}Fe incorporation in transformation ossicle, bone marrow and spleen. It was found that hypophysectomy 9 to 26 d before collection resulted in a profound decrease in erythropoiesis as revealed by ^{59}Fe incorporation (Table 2).

The results demonstrate that the onset of haematopoiesis

is critically dependent on pituitary hormones. The availability of a hormone-dependent differentiating haematopoietic system in post-foetal life makes it possible to integrate the factors regulating hormone-dependent and hormone-independent haematopoiesis.

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Depletion of L-cell sterol depresses endocytosis

THE engulfment of droplets of fluid, from the surrounding medium (endocytosis or pinocytosis), represents a phenomenon of the plasma membrane of many mammalian cells^{1–3}. The physiological significance of the process is not well understood although circumstantial evidence seems to suggest that it is necessary for normal functions of certain cell types. Steinman *et al.*⁴ have established the qualitative and quantitative aspects of endocytosis and have demonstrated that a variety of cells directly interiorise soluble proteins (for example, horseradish peroxidase) by invagination of the plasma membrane without previous binding of the proteins to cell-surface receptors. Cholesterol is a prominent constituent of the plasma membrane of mammalian cells, and its role in many complex functions of the cell membrane is just beginning to be understood. It buffers the 'fluidity' of the plasma membrane, increasing the viscosity at high temperatures and impeding the transition into the gel state at reduced temperatures^{5–7}. Through its action on membrane fluidity, or possibly more directly, it affects specific functions of the membrane such as ion transport and enzyme activities⁸. With these manifold effects of cholesterol on membrane structure and function in mind, we investigated the effect of altered sterol concentration on the ability of cultured cells to carry out endocytosis, to clarify further the role of cholesterol in membrane-dependent cytological functions.

The procedures we used to investigate the role of membrane-associated sterol in endocytosis are based on our finding that certain oxygenated derivatives of cholesterol (for example, 25-hydroxycholesterol and 7-ketocholesterol) are potent inhibi-

Table 2 Decline of ^{59}Fe incorporation in transformation ossicles, femoral bone marrow and spleen following hypophysectomy

Hypophysectomy day	^{59}Fe incorporation (c.p.m. mg ⁻¹)		
	Ossicle	Bone marrow	Spleen
	Hypophysectomised rats		
0	2 $\pm$ 0.4*	179 $\pm$ 50*	10 $\pm$ 1*
8	1 $\pm$ 0.4*	165 $\pm$ 45*	10 $\pm$ 1*
12	4 $\pm$ 2*	96 $\pm$ 39*	8 $\pm$ 1*
22	23 $\pm$ 6*	297 $\pm$ 6*	14 $\pm$ 2*
—	Control rats; no operation		
	408 $\pm$ 111	3247 $\pm$ 169	484 $\pm$ 95

Control and hypophysectomised rats were littermates. Autopsy was performed 26–31 d after transplantation of bone matrix. Hypophysectomy was performed on the days indicated. ^{59}Fe incorporation into protein-bound haem was determined as described². All values represent mean $\pm$ s.e. of 8 observations.

*Significant difference from control.

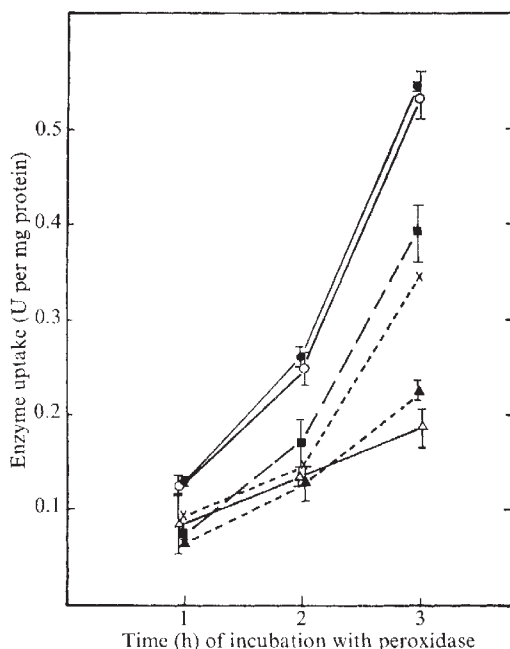


Fig. 1 Rate of endocytosis in L cells after treatment with various inhibitors of sterol synthesis. The culture medium contained 25-hydroxycholesterol $1 \mu\text{g ml}^{-1}$ (Δ), 20 α -hydroxycholesterol, $1 \mu\text{g ml}^{-1}$ ($\blacktriangle$), 7-ketocholesterol $1 \mu\text{g ml}^{-1}$ ($\blacksquare$), 6-ketocholestanol $1 \mu\text{g ml}^{-1}$ ($\times$) or cholesterol $100 \mu\text{g ml}^{-1}$ ($\bullet$). Control cultures contained 50 μl 5% bovine serum albumin (BSA) solution used to suspend the sterols ($\circ$). The cultures were incubated for 16 h at 37°C . Each point represents the mean $\pm$ s.e. of 3–5 cultures.

tors of sterol synthesis in cultured cells⁹. The inhibitory sterols specifically depress the activity of 3-hydroxy-3-methylglutaryl-(HMG)-CoA reductase, the regulatory enzyme in the sterol synthesis pathway. Treatment of L cell (mouse fibroblast) cultures with these inhibitors for periods longer than 1 d reduces the sterol (desmosterol) content of the cells and their plasma membranes to about one-half of the original level (A.A.K. and H.W.C., unpublished and ref. 10). Cells which are depleted of sterol do not grow or divide unless an exogenous, non-inhibitory sterol (cholesterol or desmosterol) or a sterol precursor (mevalonate) is supplied¹⁰.

L cells were grown in Waymouth's MB 752/1 medium supplemented as described previously⁹. The cells were seeded in 35-mm plastic dishes of 2-ml portions containing a total of 0.7×10^6 to 2×10^6 cells depending on the type of experiment. Different inhibitors of sterol synthesis (6-ketocholestanol, 7-ketocholesterol, 20 α -hydroxycholesterol, or 25-hydroxycholesterol) were added to the medium (2 ml) in 50 μl of a 5% solution of crystallised bovine serum albumin (BSA) containing 10% ethanol⁹ so that the final concentration of sterol was $1 \mu\text{g ml}^{-1}$. Control cultures received 50 μl of the albumin-ethanol solution.

Cellular endocytosis was assayed as described by Steinman *et al.*⁴. Briefly, L cells were incubated for various periods of time with sterols and/or their precursors and were then reincubated for 1–3 h with horseradish peroxidase (Sigma, type II) dissolved in fresh culture medium (1 mg ml^{-1}). The peroxidase-containing medium was removed by suction, the cultures were gently washed five times with phosphate-buffered saline, and then reincubated for 50 min at 37°C in Waymouth MB 752/1 medium to remove all non-ingested peroxidase. The cells were then lysed by incubation with an aqueous solution of Triton X-100 (0.05% v/v) for 2 h at room temperature. The amount of peroxidase in the lysate was determined as described by Steinman *et al.*⁴. These authors have shown that: there are no cell-surface receptors for the probe (peroxidase); the rate of endocytosis increases linearly with temperature; endocytosis is dependent on ATP

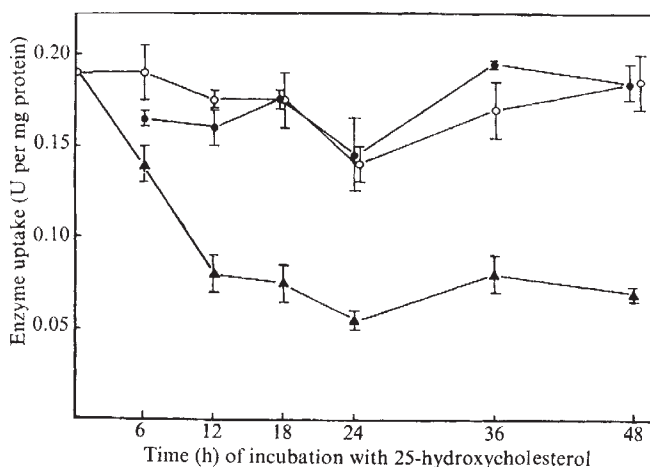
generated by glycolysis and the respiratory chain; and the process is independent of the cell cycle.

Figure 1 shows the effects of incubating various inhibitors of sterol synthesis with L cells for 16 h on the ability of the cells to carry out endocytosis. 20 α -hydroxycholesterol and 25-hydroxycholesterol depressed the rate of endocytosis by 42 and 54% respectively, whereas 6-ketocholestanol and 7-ketocholesterol were less active. The relative potencies of these sterols as suppressors of endocytosis were similar to their reported potencies as inhibitors of sterol synthesis⁹. Purified cholesterol at a high dose ($100 \mu\text{g ml}^{-1}$) had no effect on endocytosis. The rate of uptake of peroxidase in the control cultures averaged 167 ng per mg cellular protein per h. This corresponds approximately to 0.0049% of the administered peroxidase per 10^6 cells h^{-1} , a value which is slightly higher than the corresponding range of 0.0032–0.0035% reported by Steinman *et al.*⁴ in another subline of L cells.

The effect of 25-hydroxycholesterol on endocytosis as a function of time during which the inhibitor was incubated with the cultures is shown in Fig. 2. Endocytosis was quantitatively affected as early as 6 h after addition of 25-hydroxycholesterol. After 12 h the level of endocytosis had reached a minimum level which remained unchanged for the following 36 h. The slope of the curve in Fig. 2 appears to be somewhat steeper than the decline in the concentration of membrane sterol in other similar experiments and was preceded by the disappearance of HMG-CoA reductase activity and the loss of sterol synthesis (ref. 9 and A.A.K. and H.W.C., unpublished). The effect of incubating L cells for 16 h with various concentrations of 25-hydroxycholesterol is shown in Fig. 3. Endocytosis was affected by 25-hydroxycholesterol at a concentration as low as $0.05 \mu\text{g ml}^{-1}$ (10^{-7} M) and maximal depression of endocytosis was reached at concentrations of $0.5 \mu\text{g ml}^{-1}$.

L cells, in which HMG-CoA reductase activity is depressed by 25-hydroxycholesterol, are able to synthesise sterols from exogenous mevalonate, which is the product of the reaction catalysed by the reductase. In similar conditions they are not able to synthesise sterols from acetate or HMG^{9,10}. Table 1 shows that the addition of exogenous mevalonate or cholesterol resulted in the maintenance of a normal level of endocytosis in the presence of 25-hydroxycholesterol. In other experiments (not shown) acetate or HMG did not relieve the inhibitory effect of 25-hydroxycholesterol. The recovery of endocytosis with meva-

Fig. 2 Time course of inhibition of endocytosis by 25-hydroxycholesterol. The culture medium contained 5% foetal calf serum ($\bullet$), 25-hydroxycholesterol $1 \mu\text{g ml}^{-1}$ in 50 μl 5% BSA solution ($\blacktriangle$) or 50 μl BSA solution ($\circ$). After the incubation periods indicated, rates of endocytosis over a 2-h period were determined. Values shown represent the mean $\pm$ s.e. for 3 cultures.



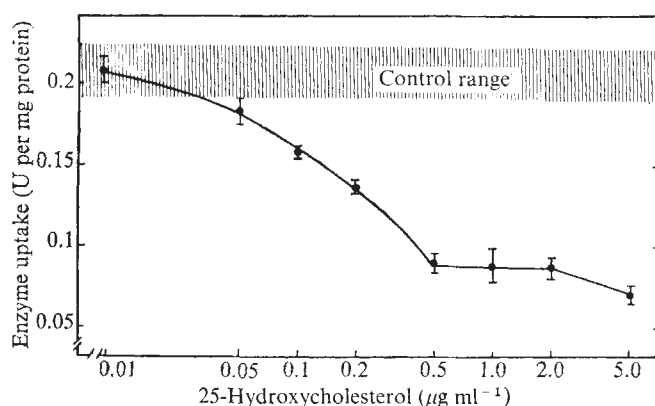


Fig. 3 Effect of incubating L cells with varying concentrations of 25-hydroxycholesterol on the rate of endocytosis. 25-hydroxycholesterol was added to the culture medium in 50 µl 5% BSA solution. After incubation for 16 h with the inhibitor, rates of endocytosis were determined over a 2-h period. Values shown represent the mean $\pm$ s.e. for 3 cultures.

lonate supports our previous findings (refs 9 and 10 and A.A.K. and H.W.C., unpublished) that the inhibitory sterols do not have effects other than those resulting from the suppression of HMG-CoA reductase and sterol synthesis.

The possibility that incubation of L cells with the inhibitory sterols may have depressed the level of intracellular ATP was examined by comparing the concentration of ATP in cultures incubated with or without 25-hydroxycholesterol. Using the luciferin-luciferase assay^{11,12}, we did not detect any significant differences in the concentration of ATP between control cultures and cultures treated with 25-hydroxycholesterol for as long as 40 h. As the cultures approached confluency the level of ATP dropped in both groups. A diminished ATP level in confluent cell cultures has been reported by others⁴.

These results, indicating that cells which are deficient in sterol are unable to carry out endocytosis at normal rates, emphasise the important functional role of cholesterol in the plasma membrane. Dianzani *et al.*¹³ have demonstrated that increased amounts of cholesterol in the plasma membrane of macrophages depresses phagocytosis—that is, endocytosis of particulate material. If phagocytosis in macrophages and endocytosis in cultured L cells are accomplished by the same basic mechanism, then our results, together with their observations, indicate that the concentration of cholesterol in mammalian cell membranes must be maintained within a fairly narrow range to ensure their

proper biological functioning. Our results may also be related to certain observations of altered functioning of fibroblasts from patients with familial hypercholesterolaemia. Since fibroblasts from these patients cannot take up low density lipoprotein (LDL) at a normal rate¹⁴ it would be of interest to determine whether or not there is any abnormal concentration of cholesterol in the plasma membranes of these cells. Our observations also suggest an alternative interpretation of data published by Brown and Goldstein¹⁵ which showed a diminished uptake of LDL in normal human fibroblasts that had been preincubated with 25-hydroxycholesterol or LDL. Since both surface-bound and internalised LDL were included in the measurement of LDL 'binding' it could be argued that the effect of 25-hydroxycholesterol was to diminish endocytosis rather than to reduce the number of LDL receptors as originally suggested by Brown and Goldstein.

It is established that cholesterol has an important role in the regulation of the activity of membrane-localised Na^+/K^+ ATPase in mycoplasma⁹ and in mammalian cells^{9,16,17}. Thus, changes in endocytosis and in ion transport both may contribute to the inhibition of DNA synthesis that we have observed in L cells and in lectin-stimulated lymphocytes treated with inhibitors of sterol synthesis (ref. 18 and A.A.K. and H.W.C., unpublished). Whether the continuous uptake of solutes and membrane material is necessary for the maintenance of normal cell functions needs further clarification. The present study suggests, however, that cholesterol is a critical functional as well as structural unit in the plasma membrane and that its depletion results in the modification of specific membrane-dependent cellular functions.

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Table 1 Rate of endocytosis in L cells

Additions*	Peroxidase uptake† (ng per mg cellular protein per h)	% Control	t test
Control	242.1 $\pm$ 29.5 (7)	100	
25-Hydroxycholesterol (1 µg ml ⁻¹)	109.0 $\pm$ 26.9 (7)	45	$P < 0.01$
25-Hydroxycholesterol (1 µg ml ⁻¹) + mevalonic acid (0.5 mg ml ⁻¹)	229.2 $\pm$ 20.8 (2)	95	NS
25-Hydroxycholesterol (1 µg ml ⁻¹) + cholesterol (100 µg ml ⁻¹)	250.0 $\pm$ 27.8 (2)	103	NS

*Cells were cultured for 36 h in the presence of the listed compounds.

†The rate values (mean $\pm$ s.e. for 3–4 cultures) were based on measurements obtained between the first and second hour of incubation with horseradish peroxidase. Numbers in parentheses indicate the number of experiments. The t test results indicate the probability of a significant difference between control and experimental values.

Duplication of biochemical changes of Huntington's chorea by intrastriatal injections of glutamic and kainic acids

SYSTEMIC administration of large doses of L-glutamate to immature animals causes degeneration of neurones in the retina and the arcuate nucleus. Olney *et al.*¹ reported that intracerebral injections of the more potent excitatory analogues of glutamate, in particular kainic acid, caused rapid degeneration of certain dendritic and somal structures in the injected area. Presumably the degeneration is related to the ability to bind the "excitotoxic" agent, with necrosis resulting from a sustained increase in membrane permeability.³ H-kainic acid binds more strongly to striatal tissue than to any other area of rat

brain tested². This lends some support to Spencer's theory³—based on antagonism of cortical excitation of striatal neurones by diethyl glutamate—that the massive corticostriatal pathway is glutaminergic. If Huntington's chorea were an excitotoxic phenomenon resulting from chronic overstimulation of glutamate receptors, striatal neurones in particular would be affected because of heavy glutaminergic input. This hypothesis is not readily tested by simple measurement of glutamate in choreic tissue because glutamate is a precursor of γ -aminobutyric acid (GABA) and is also involved in other metabolic activities. As one potential test of this hypothesis, we have injected 1 μ l of either kainic acid or L-glutamic acid dissolved in isotonic saline directly into the extrapyramidal nuclei of rats. Injection into the striatum produced local enzymatic changes duplicating those reported in Huntington's chorea.

The sites of injection and the coordinates by the Konig and Klippel atlas⁴ were: caudate, AP 8.38, L 2.8, DV +0.2; ventrolateral thalamus, AP 4.75, L 2.8, DV +1.4; and substantia nigra, AP 2.18, L 2.1, DV -2.2. Injections were into 300-g male Wistar rats under sodium pentobarbital anaesthesia.

The extrapyramidal system has neurones containing dopamine, acetylcholine and GABA. The state of these neurones in extrapyramidal disorders such as Huntington's chorea and Parkinson's disease is reflected by the level of the enzymes for transmitter synthesis⁵. Therefore we measured the levels of tyrosine hydroxylase (TH), choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) in the rat brain extrapyramidal nuclei after the injections.

After 2 or 3 d, the rats were killed by cervical fracture, the brains were removed rapidly, and the desired nuclei were dissected and homogenised in 0.25 M sucrose. Aliquots of these sucrose homogenates were used for enzyme determinations by the radioactive methods used in the study of enzyme activities in human brain tissue⁵. Protein was determined by the Folin-Ciocalteu method.

The biochemical results are shown in Tables 1 and 2. After injections of 5 nmol of kainic acid (Table 1), the largest changes were observed with striatal injections. There were large losses of GAD and CAT (to 34% and 36%, respectively) in the striatum itself, and there was a smaller decrease of GAD in the substantia nigra (to 66%). There was also a significant increase (to 128%) in striatal TH. With comparable thalamic injections, there were no significant enzyme changes in the substantia nigra and caudate, and only a mild drop in thalamic GAD. Nigral injections also produced no significant striatal changes, although there was a slight drop in nigral TH.

When the amount of kainic acid injected into the caudate was reduced to 2.5 nmol, the effect was reduced, and 1 nmol had no effect. A dose of 50 nmol of L-glutamic acid produced the equivalent effect of 2.5 nmol of kainic acid (Table 2).

Table 1 Some enzyme activities on the side injected with 5 nmol of kainic acid as a percentage of those on the control side

Area injected		Area analysed	
Enzyme	Striatum	Thalamus	Substantia nigra
Caudate (6)			
GAD	34 $\pm$ 3%*	107 $\pm$ 16%	66 $\pm$ 14%†
CAT	36 $\pm$ 8%*	113 $\pm$ 16%	119 $\pm$ 21%
TH	128 $\pm$ 9%†	91 $\pm$ 12%	100 $\pm$ 14%
Thalamus (3)			
GAD	90 $\pm$ 15%	72 $\pm$ 11%†	96 $\pm$ 6%
CAT	109 $\pm$ 12%	114 $\pm$ 15%	—
TH	94 $\pm$ 11%	92 $\pm$ 14%	—
Substantia nigra (4)			
GAD	107 $\pm$ 9%	106 $\pm$ 9%	125 $\pm$ 27%
CAT	90 $\pm$ 10%	71 $\pm$ 8%†	107 $\pm$ 19%
TH	87 $\pm$ 14%	70 $\pm$ 20%	73 $\pm$ 9%†

Control values were the same as found in uninjected animals and averaged for the striatum (in μ m per h per 100 mg protein): 15.5 for GAD; 31.3 for CAT and 0.175 for TH. Protein content was not significantly different in the injected as compared to the control samples. Data given as mean $\pm$ s.e., number of animals in parentheses.

* $P < 0.001$.

† $P < 0.05$.

Table 2 Enzyme levels in striatum on injected side as percentage of those on contralateral side

Material injected	CAT	GAD	TH
Kainic acid, 2.5 nmol	81 $\pm$ 4%*	84 $\pm$ 6%*	111 $\pm$ 8%
Kainic acid, 1 nmol	100 $\pm$ 8%	91 $\pm$ 7%	113 $\pm$ 12%
L-glutamic, 50 nmol	82 $\pm$ 5%*	83 $\pm$ 4%*	114 $\pm$ 6%

Figures are mean $\pm$ s.e. Four rats were used in each case.

* $P < 0.05$.

The reduction in striatal CAT, and striatal and nigral GAD, with sparing or enhancement of TH, parallels the biochemical findings in Huntington's chorea⁵⁻⁷. Thus, these excitotoxic injections produced a biochemical model of the disease. They also produced marked physical manifestations.

Rats injected with 5 nmol of kainic acid into the left caudate almost immediately developed diarrhoea and stopped grooming. By the third day, they were bleeding from their nose and urinary tract. When injections were made in the thalamus there was similar, though lesser bleeding, and loss of grooming. Injections into the substantia nigra did not cause bleeding but did cause the mouth and eyes to water. Rats given the injections in either the left caudate or left substantia nigra held their heads tilted markedly to the left with their bodies hunched. Rotation was not prominent, however, except when injections were made into the thalamus: then each rat, on awaking from the anaesthesia 30–60 min after injection, rolled over and over slowly to the left while the tail was held stiffly erect and rotated clockwise like a propeller and the head was tilted to the left. After 2 or 3 h rolling ceased and the animal alternately stood erect and pirouetted, also clockwise. Subsequently, the rat repeatedly circled the plastic cage in a clockwise direction, rubbing against the wall and with the head still tilted to the left. These movements had stopped by the next day.

Rats injected with 1 or 2.5 nmol of kainic acid or with 50 nmol of L-glutamic acid into the caudate showed no dramatic effects.

With the 5-nmol injections of kainic acid, two of eight caudate-injected rats died between the second and third day, as did three of six thalamus-injected rats. There were no deaths observed in the substantia nigra-injected rats. All three rats that received this dose of kainic acid in the globus pallidus died within 24 h.

The injections were always unilateral and no behavioural tests were carried out. To establish whether the excitotoxic model of Huntington's chorea truly parallels the disease, further exploration of the motor and behavioural effects is needed.

The excitotoxic hypothesis of Huntington's chorea might also help to explain the phenomenon of tardive dyskinesia. This motor disorder is characterised by choreiform movements which are hard to distinguish from Huntington's chorea and which appear after long term therapy with dopaminergic blockers. The dopaminergic nigro-striatal tract is believed to be inhibitory towards striatal interneurons, and administration of neuroleptics apparently releases cholinergic interneurons^{8,9}. This removal of inhibition could result in normal excitatory input from the cortex, possibly of a glutaminergic nature, becoming excitotoxic and gradually destroying striatal interneurons.

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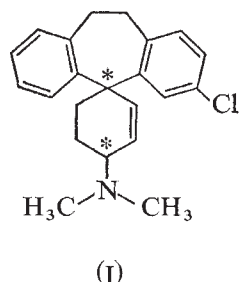
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Stereoselective effects of the potentially neuroleptic rigid spiro amines

WE HAVE reported the synthesis and pharmacological effects of a novel series of tetracyclic spiro compounds structurally related to the tricyclic antidepressants¹. In contrast to the common tricyclic antidepressants, the terminal amino group of the spiro compounds has restricted mobility. As expected, some of these compounds have antidepressant properties in model systems, for example they are potent blockers of the neuronal uptake of noradrenaline in the central nervous system. But the compounds containing a chlorine atom in the tricyclic moiety have a neuroleptic profile in animal tests. 3-Chloro-10,11-dihydro-N,N-dimethylspiro-[5H-dibenzo[*a,d*]cycloheptene-5,1'-cyclohex-2'-ene]-4'-amine, structure (I), is particularly interesting in this respect. The cyclohexene ring in this structure is located perpendicularly to the tricyclic ring system and as a consequence the amino group can occupy a position either *cis* or *trans* to the chlorine-containing part of the tricyclic ring system. Furthermore, the *cis* and *trans* isomers can be resolved into enantiomers as the molecule contains two chiral centres (marked with asterisks in structure (I)). Several studies have yielded re-



sults to favour the view that neuroleptic drugs act as dopamine (DA) antagonists in the brain^{2,3} and that the DA receptor blockade is rather well correlated with their antipsychotic efficiency^{4–6}. The neuroleptics seem to block chiefly postsynaptic DA receptors, particularly in striatal⁷ and mesolimbic⁸ DA areas of the brain. The rigid nature of the isomeric tetracyclic spiro amines of structure (I), and the possibility of stereospecific pharmacological effects make these compounds interesting tools for an investigation of the structural requirements for DA receptor blockade and for studies on DA receptor topology.

The *cis* and *trans* isomers have now been separated in the following way. A substance, melting point 245–246 °C (hydrochloride), prepared essentially as described previously¹, was converted to the corresponding oxalate. Fractional crystallisation of this oxalate gave partial separation. Conversion to the hydrochlorides and renewed crystallisation resulted in the pure isomers. These are referred to as the α isomer (melting point 257–259 °C (hydrochloride)), and the β isomer (melting point 226–228 °C (hydrochloride)).

The separation was monitored by nuclear magnetic resonance spectroscopy. Comparison of the spectra of the pure isomers with that of the substance used in our previous

Table 1 Effects on apomorphine stereotypies in the rat

Compounds	Reg. no*	ED ₅₀ ($\mu\text{mol kg}^{-1}$)
$\alpha + \beta$	A 02056	40
α	A 23622	> 54
β	A 23623	10
(+)- α	A 23885	> 54
(-)- α	A 23886	> 54
(-)- β	A 23887	4
(+)- β	A 23888	> 44
Chlorpromazine		14

*Astra's internal code number.

Apomorphine (2 mg kg⁻¹) was administered intraperitoneally to male Sprague-Dawley rats (150–200 g). The test compounds were injected intraperitoneally 30 min before apomorphine in five doses (six animals per dose). The stereotypies were scored according to Costall and Naylor¹¹. ED₅₀ refers to the dose of the test compounds which reduces the stereotypies by 50% 20 min after apomorphine administration.

work¹ showed that the latter consisted of a mixture of about equal parts of α and β isomers.

The α and β isomers have been resolved using the optically active mandelic acids and di-*p*-toluoyltartaric acids. The antipodes of the two isomers have specific rotations of 148° (hydrochloride in ethanol) and 204° (maleate in chloroform), respectively.

The isomers were evaluated for their ability to inhibit apomorphine-induced stereotypies in the rat; this is a selective and sensitive test for neuroleptic activity⁹. Apomorphine stereotypies seem to be a result of DA receptor stimulation in the brain, which makes this test an *in vivo* technique for measuring DA receptor blockade¹⁰.

The results of the apomorphine test show differences in activity between the isomers (Table 1). The *cis-trans* mixture of the spiro amine (I) is a potent blocker in this test. The pharmacological activity resides chiefly in the β isomer which is about as potent as chlorpromazine. Studies on the enantiomers revealed a further difference in activity. Only one of the enantiomers, (–)- β , was found to be active in this test, being three or four times as potent as chlorpromazine. Further evidence for the specificity of this enantiomer as a blocker of the DA receptor was provided by DA turnover studies in the rat. It is well known that neuroleptic drugs accelerate DA turnover, probably due to an increase in DA neuronal activity as a result of DA receptor blockade^{7–9}. Of the two pairs of enantiomers, only the (–)- β isomer increased DA turnover in the brain.

In studies of thioxanthenes, the *cis* isomers, were found to be considerably more potent than the corresponding *trans* isomers in various tests for neuroleptic activity^{12,13}. It may therefore be postulated that the active tetracyclic spiro amine (–)- β has the *cis* configuration. The stereochemistry of the isomers is being investigated by X-ray diffraction methods.

We conclude that there are strict stereochemical requirements for a potent and selective blocker of the DA receptor. The compounds discussed here apparently fulfil these requirements.

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Ca²⁺-dependent arrest of cilia without uncoupling epithelial cells

IN CERTAIN ciliated epithelia, for example, mussel gill lateral cell epithelium, metachronally coordinated ciliary beat can be arrested systematically, either after nervous¹ or local stimulation, for example by laser microinjury^{2,3}. In the latter case, the velocity of spread of arrest, its extent and decremental character suggest that the response depends on electrotonic coupling of the gill cells³. The lateral cells are coupled by extensive septate junctions and small gap junctions⁴, more basally located, which may act in a manner homologous to electrical synapses of nerve and muscle cells⁵⁻⁷. Although intracellular microelectrode recordings of lateral cell depolarisation accompanying arrest after branchial nerve stimulation have been made in *Mytilus*⁸, there are no reports of microelectrode studies of cell coupling in gill cells. We have used experimentally-induced spreading arrest to monitor the state of coupling in the lateral cell epithelium of a freshwater mussel (for example, *Elliptio complanatus*).

Lateral cell cilia are arrested by an increase in cytoplasmic calcium ion concentration. Arrest does not spread past a focus of local damage in *Mytilus* gill in the absence of Ca²⁺ in the bathing medium³. Furthermore, when gill filaments from freshwater mussels are placed in 12.5 mM CaCl₂, the cilia beat metachronally; when a divalent cation ionophore A23187 is added, arrest occurs; arrest does not occur by ionophore in the presence of Na, K⁺ or Mg²⁺, without Ca²⁺ (ref. 8).

An increase in local Ca²⁺ concentration causes coupled epithelial cells of *Chironomus* salivary gland to uncouple⁹, but Rose and Loewenstein¹⁰ have shown that Ca²⁺ influx into a cell is often quenched by various cell components, so that Ca²⁺ concentration in the cytoplasm may change only minimally, if at all, in parts of the cell distant from the site of influx. It may therefore be that even when Ca²⁺ is entering a cell at the apical surface, the effective concentration of Ca²⁺ at the cell junctions remains low and the cells remain coupled. This seems to be a necessary conclusion in many circumstances, for example, during cardiac

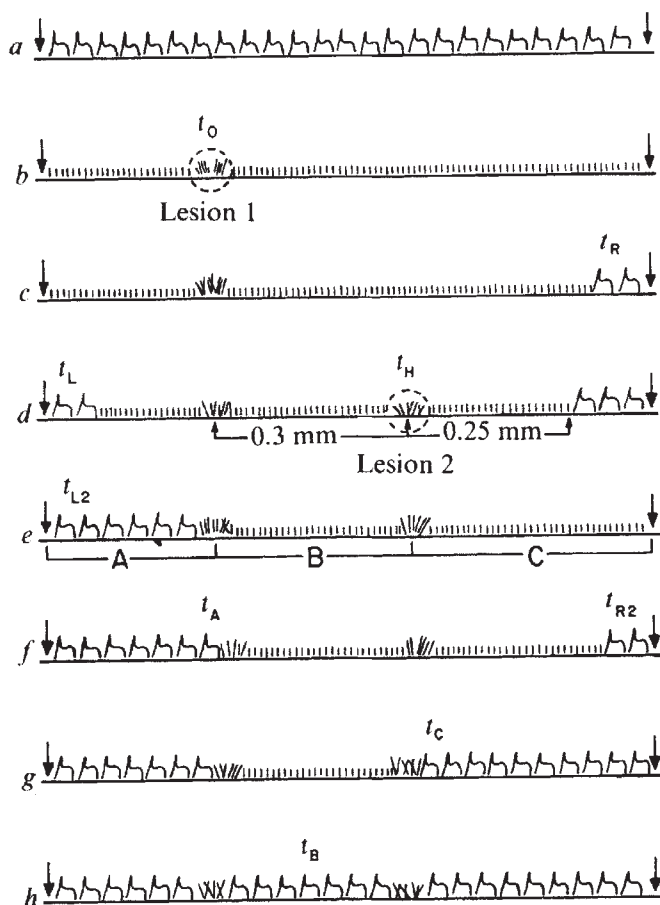


Fig. 1 Design of summation experiment. A gill filament beating with good metachronal waves (η -like structures) selected (a). At $t = 0$, a laser hit (lesion no. 1) made; within a few seconds along a defined field (length about 1 mm, arrows) metachronism ceased (b). The initial recovery to either side of the field was measured (t_R and t_L) (c). The gill was repositioned so that a second hit (lesion no. 2) could be made about 0.25 mm away from a returning wavefront (d). At t_H , this hit divided the gill filament into regions A, B and C (e). The effect on recovery was measured by the reappearance of metachronism to either side of the field (t_{L2} and t_{R2}). The geometry varied slightly in individual experiments. Usually region A recovered completely (t_A) (f); then region C (t_C) (g) and finally region B recovered (t_B) (h) with a time course given by equation (3) (see text). Cells damaged by the hits never recovered.

muscle contraction, but as far as we know it has not been demonstrated precisely with any system. We report here that mussel gill cells remain coupled during ciliary arrest.

Gills of freshwater mussels (Carolina Biological Supply Co., Gladstone, Oregon) were excised and placed in 12.5 mM CaCl₂; they usually remained fully active for many

Table 1 Typical arrest and recovery parameters after laser microinjury

Experiment no.	R	L	H	L ₂	Time (s)			A	C	0.63A+(C-H)	B
					L _{2-H}	R ₂	R _{2-H}				
1	23	32	40	40	0	80	40	93	125	144	140
2	20	15	26	26	0	52	26	58	75	86	87
3	8	7	10	10	0	20	10	12	115	113	115
4	13	5	14	14	0	25	11	79	52	88	97
5	10	8	11	11	0	33	22	20	60	62	60
6	55	50	56	56	0	90	34	117	105	123	155
7	61	60	64	110	46	99	35	150	105	135	129
8	25	70	40	—	—	58	18	105	115	141	160
9	44	75	50	—	—	95	45	90	115	122	115
10	7	17	9	—	—	15	6	28	24	33	31
Average*	24.7	34.4	27.8	—	—	54.3	26.5	71.1	78.5	95.5	103.3
s.d.	22.9	24.6	23.8	—	—	36.0	18.5	39.4	42.5	36.1	41.3

*31 experiments, t test: A compared with B, $P < 0.001$; C compared with B, $P < 0.01$; (0.63A+C-H) compared with B, $P > 0.3$.

hours with almost 100% metachronism. Small pieces of active gill were placed on a depression slide containing three or four drops of 1% blue dye FDC No. 1 in CaCl_2 , for laser microdisruption as described before^{2,3,11}. A pulsed ruby laser microbeam was focused near the base of the beating cilia at a selected point to produce a focal lesion of about 30 μm diameter (Fig. 1a and b). The timing of all subsequent events (Table 1) was measured from the moment of microbeam irradiation (t_0).

The lesion induced arrest of ciliary beat which spread, as expected, along the gill filament up to about 1 mm on either side of the hit (Fig. 1b)^{2,3}. Characteristically, metachronism was first reactivated in the cells most distant from the hit. The time that metachronism reappeared in a defined field to either side of the lesion (t_R and t_L) was recorded (Fig. 1c and d). Between the original lesion and the returning metachronal wave cilia were still arrested. The gill was then moved so that the laser microbeam was focused near the centre of the arrested field to one side of the lesion, approximately 0.25 mm away (10 cell lengths) from the returning metachronal wavefront, so as to produce a second lesion at this point (Fig. 1d). The gill filament was thus divided into three regions: A, B, and C, where B lies between the two lesions, and C is beyond the second lesion (Fig. 1e).

In about 90% of experiments, the second lesion reintroduced arrest in region C (Fig. 1e). Experimental failure probably reflects our inability to focus the laser accurately and the consequent variability of microbeam strength at lateral cell sites. This also accounts for much of the variability in the data, shorter times probably reflecting weaker hits. We measured the time after the second hit when metachronism first reappeared in region C (t_{R2-H}) (Fig. 1f). Typical data are given in Table 1; recovery was delayed on the average by 26.5 s (31 experiments).

To produce such delay, the stimulus produced by the second lesion must apparently have spread through an average of 10 lateral cells whose cilia are arrested, before reaching the reactivating cells where we observed secondary arrest. This supports the conclusion that the cells with arrested cilia were still coupled, but does not rule out an alternative possibility that cells (for example, the intermediate cells) adjacent to the column of arrested lateral cells provided the coupling pathways, being coupled to one another and to the reactivating cells at the time of the second hit.

In contrast, the second lesion reintroduced arrest in region A, beyond the first lesion, in only 20% of experiments; that is, recovery (t_{L2-H} ; Table 1) is not delayed (Fig. 1e). We interpret this to mean that lesion No. 1 usually destroyed the physical integrity of the gill filament, so that the column of cells beyond the lesion to the left was uncoupled from the column of cells to the right. Therefore, the stimulus produced by the second lesion should have affected the cilia of regions B and C but not those of region A. If this is correct, the probability is greatly reduced that coupling that affects the lateral cell metachronism can occur through an alternate pathway adjacent to the lateral cells.

We have demonstrated conclusively that cells with arrested cilia are still coupled, at least as defined by the ability to respond to a second distant stimulus, by measuring the times for complete recovery in regions, A, B and C (t_A , t_B and t_C , respectively; Fig. 1f, g and h). If arrested cells are uncoupled, we would expect recovery in region B to have been unaffected by the second lesion so that t_A and t_B would have been approximately equal. This was not the case (Table 1). If arrested cells are coupled, then region B would have been affected by the second hit, so that $t_B > t_A$ because of the induced recovery delay. Two simple explanations consistent with coupling were that (1) t_B and t_C are equal or (2) that region B would have recovered as if the hits were additive.

The first laser hit presumably produces a stimulus that enables sufficient Ca^{2+} from the bathing fluid to enter the apical surfaces of cells near the lesion to induce ciliary arrest in sequential fashion. Our results demonstrate that the pathway of coupling remains functional after ciliary arrest, although it is readily interrupted by a break in epithelial integrity. Our best explanation of the summation effect in the region between the two hits is that (1) the second hit produces further Ca^{2+} influx into the coupled chain of cells with arrested cilia and that (2) the recovery times measured depend on Ca^{2+} being pumped out of the cells at a constant rate such that when free Ca^{2+} falls below a critical concentration, metachronism reappears. If regions B and C are of approximately equal lengths and the stimulus is exponentially decremental with a decay constant of about 0.3 mm, then we would expect that

$$t_B = t_A + (\text{effect of second lesion}) \quad (1)$$

and that the effect of the second lesion is related to t_C in that

$$t_C - t_H = (\text{effect of second lesion}) + 0.37 t_A \quad (2)$$

so that (3)

$$t_B = 0.63 t_A + t_C - t_H \quad (3)$$

Both in individual experiments and on average ($P > 0.3$) equation (3) seems empirically valid (Table 1). This explanation supports and extends findings of Rose and Loewenstein¹⁰ in that it requires the actions of Ca^{2+} in these conditions to be restricted largely to those in the immediate locale of the point of influx of the ion into the cytoplasmic matrix of the cell, that is to the cilia and not to the junctions.

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Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*

PHOTOPERIOD governs reproduction in many insect species with adult diapause. The nervous and neuroendocrine systems mediate the impact of the environment on ovarian functions. It has been suggested that the daylengths promoting diapause prevent secretion of the activation hormone (most probably a complex of neurohormones) from the pars intercerebralis (PI) of the brain. Without this hormone the corpora allata (CA) do not secrete the gonadotropic hormone and reproduction is arrested¹⁻³. It has been shown, however, that other physiological conditions such as pregnancy⁴, fasting⁵, or lack of mating⁶ produce an inhibitory effect of the brain on the CA through the nervi allati. The inhibition is probably mediated by a nervous centre situated close to the neurosecretory cells of the PI. We show here that the photoperiod that promotes diapause arrests reproduction by a double mechanism, the

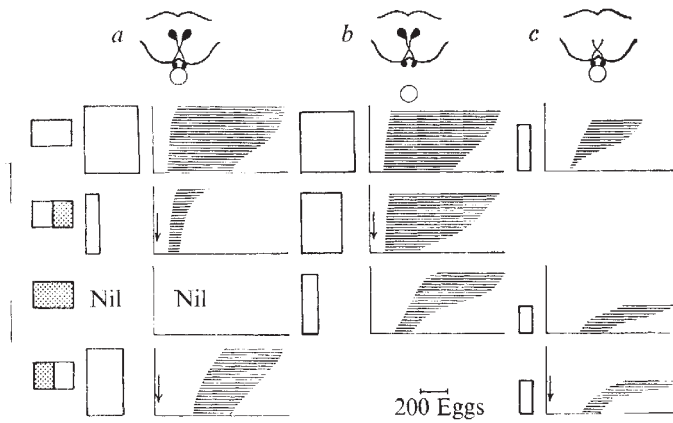


Fig. 1 Schematic representation of the effect of denervation of corpora allata and extirpation of pars intercerebralis on reproductive activity. The first column of oblongs indicates photoperiods. Open, long day (LD 18 : 6); shaded, short day (LD 12 : 12). The three series of oblongs and graphs represent: *a*, sham operations ($n = 20, 13, 35, 30$); *b*, transections of nervi allati ($n = 28, 17, 18$); *c*, extirpations of pars intercerebralis ($n = 10, 25, 44$). Oblongs represent fecundity; width, eggs per female; height, % ovipositing females. Shaded area denotes length of oviposition period in days; horizontal axis, % ovipositing females (length of axis = 100%), vertical axis, time (length of axis = 50 d). Arrows, change of photoperiod.

suppression of humoral stimulation and the inhibition of the CA through the nerves. The effect of photoperiod is mediated by the pars intercerebralis.

We have studied the role of the nervi allati and centres in the PI in the regulation of the secretory activity of the CA in females of *Pyrhocoris apterus*, under long- and short-day regimes. This species exhibits a facultative diapause controlled very regularly by the photoperiod; long daylengths have a stimulatory effect on the CA and reproduction, whereas short daylengths are inhibitory. This enables us to study both the stimulatory and inhibitory mechanisms regulating the activity of the CA. Experimental females were reared individually in Petri dishes on linden-seed at $26 \pm 2^\circ \text{C}$ and in long-day (18:6 h of light : darkness) conditions or short-day (12:12) conditions respectively. Transection of the nervi allati and extirpation of the PI were performed through an incision of the neck membrane⁷. Since size is not a reliable sign of CA activity in *P. apterus*⁸, the activity of the CA was evaluated by the rate of oviposition.

In females kept permanently under long-day conditions the denervation of the CA did not affect reproduction and therefore the activity of the CA. The same operation disturbed the inhibitory effect of short daylengths however. The control (sham-operated) females stopped oviposition soon after the transfer from long- to short-day conditions, or did not begin to oviposit when reared from the eggs at short daylengths. In the first case, transection of the nervi allati resulted in a considerable prolongation of the oviposition period and increased fecundity. In the second case, the females with denervated CA had at the least low reproductive activity (Fig. 1*a, b*). This demonstrates that the inhibitory signal of short daylengths arrives at the CA through the nervi allati. It is interesting that transection of the nervi allati in diapausing *Leptinotarsa decemlineata*¹ or *Anacridium aegyptium*³ had no effect. Transection of the nervi allato-suboesophagealis slightly activated the CA in *L. decemlineata*, but this activation was not sufficient to induce oviposition¹.

Extirpation of the PI in *P. apterus* resulted in low reproductive activity regardless of the photoperiod used (Fig. 1*a, c*). Since sham-operated females were very fecund under long-day conditions and they did reproduce under short-day conditions, the PI seems to mediate the optimal stimulation of reproduction

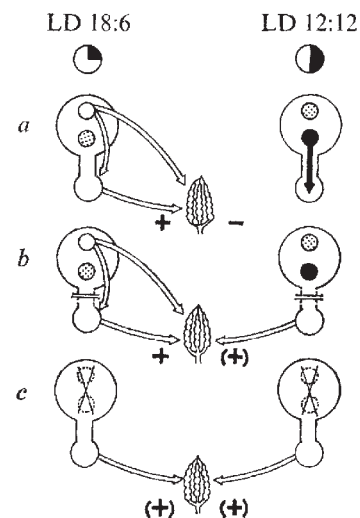
by long daylengths and also its complete inhibition by short daylengths.

In long-day conditions the PI stimulated reproduction not only through the CA but also by other pathways; the treatment of females without PI with a juvenile hormone analogue accelerated the maturation of oocytes in the first reproductive cycle, but it did not increase the overall fecundity⁹. As denervation of the CA did not affect reproduction in long-day conditions we assume that the PI stimulates the CA by way of the haemolymph. In short-day conditions, however, the PI seems to inhibit the CA through the nerves; denervation of the CA stimulated reproduction in a similar way to the extirpation of the PI. Similar rates of reproduction in both these cases also indicate that at short daylengths the PI does not exert the stimulatory effect as observed at long daylengths. The reason why denervated CA are more active in long-day females than in short-day females¹⁰ can probably be explained by the lack of humoral stimulation from the PI in short-day conditions.

The findings described enable us to propose a hypothetical model of photoperiodic regulation of reproduction in *P. apterus* (Fig. 2). According to this model the PI contains both stimulatory and inhibitory centres. The photoperiod regulates the relative activities of these two centres. In long-day conditions activation of the stimulatory centre predominates which results in a highly active CA and reproduction. Conversely, in short-day conditions the activity of the inhibitory centre prevails, the CA are inactivated and females do not oviposit at all (Fig. 2*a*). The denervation of the CA does not affect the reproductive activity of long-day females for the stimulation of the CA is humoral. The same operation, however, induces oviposition in short-day females, because the inhibitory signal does not arrive at the CA through the sectioned nervi allati. Reproductive activity is low as the action of the stimulatory centre is suppressed by short daylengths (Fig. 2*b*). Both the stimulatory and inhibitory centres are removed by the extirpation of the PI. This results in a low baseline reproductive activity for both photoperiods (Fig. 2*c*).

The common belief that the inactivity of the CA and the arrest of reproduction are caused by the diapause-promoting photoperiod only through an absence of the activation hormone

Fig. 2 Hypothetical model of photoperiodic regulation of reproduction in *P. apterus*. *a*, Intact females; *b*, females with nervi allati transected; *c*, females with pars intercerebralis extirpated. Large upper circle, brain; smaller circle below, corpora allata. Small circles inside the brain, regulatory centres; open, active stimulatory centre; solid, active inhibitory centre; shaded, inactive stimulatory or inhibitory centre. Open arrows, stimulatory action; solid arrow, inhibitory action. Effect on ovaries: +, high reproductive activity; (+), low reproductive activity; —, no reproductive activity.



cannot thus apply to all insects. In some species at least, reproductive inactivity is assured by a double mechanism—not only by the absence of humoral stimulation but also by the inhibition of the CA through the nerves. Such evidence has been obtained in *P. apterus*⁹ and in *Locusta migratoria*¹¹. A more detailed description of the present results will be given elsewhere.

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TRH potentiates excitatory actions of acetylcholine on cerebral cortical neurones

ANTAGONISM of barbiturate-induced sleeping time in experimental animals¹ is one of the interesting pharmacological properties of thyrotropin-releasing hormone (TRH; pyroglutamyl-histidyl-proline amide) which are independent of its effects on endocrine function. Moreover, this effect of TRH is

reduced or inhibited by atropine, suggesting that cholinergic mechanisms contribute to some of the effects of TRH (ref. 1). It can be shown that general anaesthetics (barbiturates in particular) selectively reduce the sensitivity of cerebral cortical neurones to the excitatory actions of iontophoretically applied acetylcholine^{2–4}. This anaesthetic interference with cholinergic, muscarinic excitations has been postulated to participate in the regulation of levels of consciousness⁵. In view of these considerations it might be predicted that TRH would alter the effects of acetylcholine (ACh) on cortical neurones and the experiments described below were designed to test this hypothesis. The results indicate that TRH potentiates the excitatory actions of ACh on cortical neurones.

Female rats (200–210 g, Charles River) were anaesthetised with pentobarbital sodium (50 mg kg⁻¹) with supplemental intraperitoneal injections when required. A small burr hole was drilled over the somatosensory cortex and after reflection of the dura the exposed cortex was covered with 0.5% agar to prevent drying. Neuronal activity was recorded by conventional means through the use of multibarrelled glass micropipettes (tip diameter, 5–10 µm), the centre barrel being filled with 2 M NaCl. The outer barrels of the electrodes were filled immediately before use by centrifugation with solutions of acetylcholine chloride (0.2 M), Na glutamate (0.5 M), carbamyl choline chloride (carbachol, 0.2 M), and TRH (0.05 M in 165 mM NaCl). Automatic current balancing through a barrel containing 2 M NaCl was used to reduce current artefacts.

Spontaneously active neurones, located between 750 µm and 1,250 µm from the cortical surface were examined since the muscarinic nature of ACh excitations of cells in this region of the cortex is well documented⁶. Applications of TRH (ejected with anodal current, 10–70 nA, 30 s–4 min) consistently enhanced the action of iontophoretically applied ACh (10–40 nA, 15–60 s). Thus, on 16 of 18 cells tested, TRH either poten-

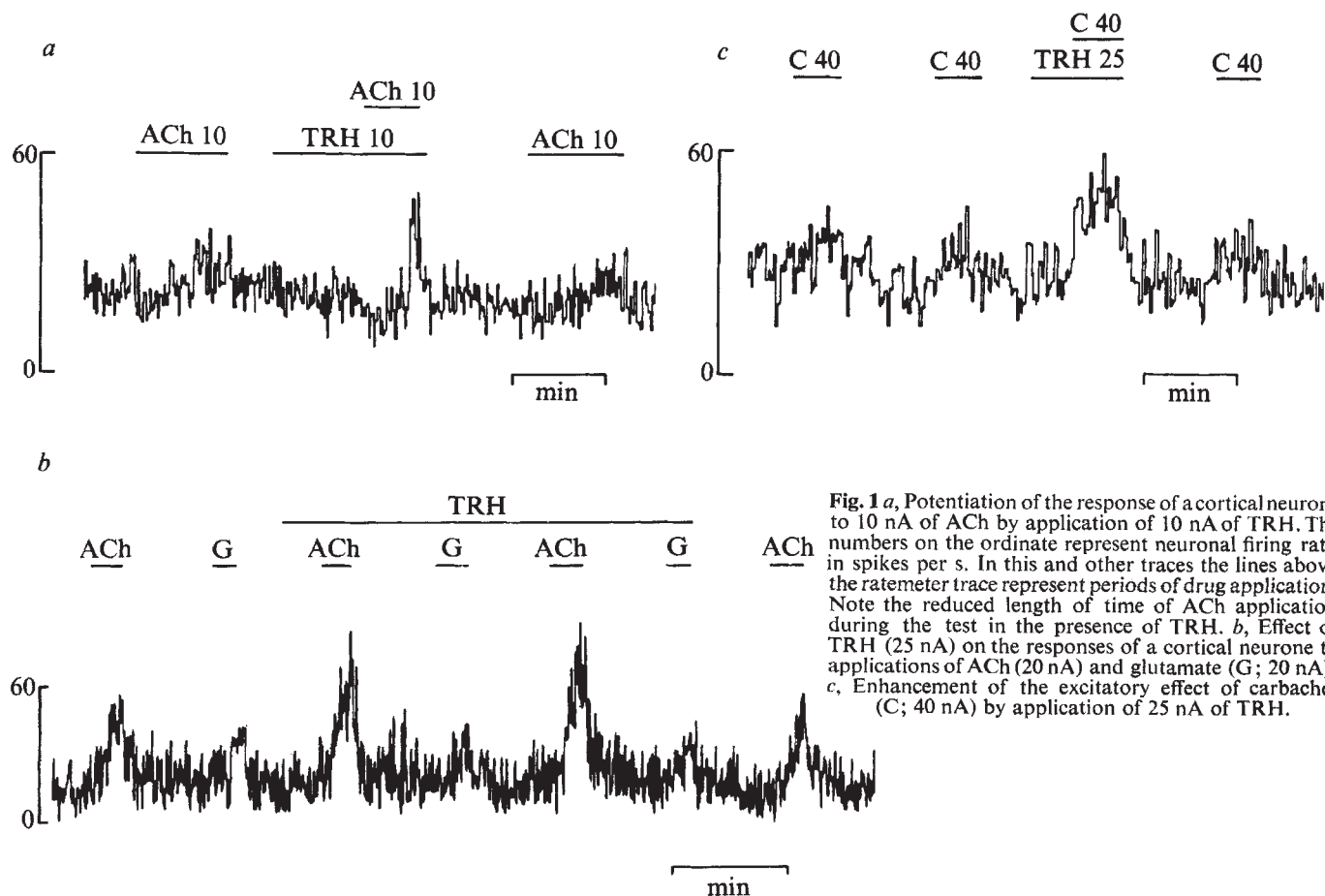


Fig. 1 *a*, Potentiation of the response of a cortical neurone to 10 nA of ACh by application of 10 nA of TRH. The numbers on the ordinate represent neuronal firing rate in spikes per s. In this and other traces the lines above the ratemeter trace represent periods of drug application. Note the reduced length of time of ACh application during the test in the presence of TRH. *b*, Effect of TRH (25 nA) on the responses of a cortical neurone to applications of ACh (20 nA) and glutamate (G; 20 nA). *c*, Enhancement of the excitatory effect of carbachol (C; 40 nA) by application of 25 nA of TRH.

tiated the excitatory actions of regularly applied pulses of ACh or converted an inactive ejection current of ACh to a typical ACh excitation. An example of this phenomenon is illustrated in Fig. 1a. TRH itself did not directly alter the excitability of the cells tested, nor did it seem to modify substantially the response of 11 cells tested with 20–50 nA of glutamate. Figure 1b shows a cell in which the response to ACh was clearly enhanced during the application of TRH while not obviously affecting the response to repeated, submaximal applications of glutamate. Since a possible explanation for the ability of TRH to enhance ACh excitations while not affecting glutamate responses might be that of acetylcholinesterase inhibition, the interactions of TRH and carbachol (not subject to degradation by acetylcholinesterase) were examined. On 11 of 13 cells tested, TRH potentiated the excitatory actions of carbachol (10–40 nA) and a typical example is shown in Fig. 1c. This effect of TRH on excitations produced by both ACh and carbachol was consistently brief in onset (sometimes occurring when the TRH and cholinergic agonist were applied simultaneously) and offset.

TRH and other peptides are being increasingly investigated with regard to their possible roles in neural function. The regional and widespread distribution of TRH throughout the neuroaxis^{6,7}, coupled with the demonstration of a depressant effect of TRH on neurones in a variety of areas^{8,9} within the central nervous system (CNS), indicates that TRH might subserve a transmitter function. TRH was not observed to have any direct effects on neuronal excitability of the restricted population of cells sampled in the present study with the ejection currents used, although it clearly and consistently enhanced the excitatory actions of ACh and carbachol. These findings may provide some insight at the neuronal level for the significant anti-anaesthetic effects of TRH in the light of the above considerations, and furthermore, may provide the basis for new approaches in attempts to understand the functional significance of TRH in the CNS. Thus, although not discounting a possible role of TRH as an intercellular mediator, the hormone may subserve a more indirect function to modulate the actions of certain chemical transmitters.

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Incision of ultraviolet-irradiated DNA by extracts of *E. coli* requires three different gene products

In most organisms pyrimidine dimers induced in DNA by ultraviolet light are removed by excision which is initiated by a repair-specific endonuclease that recognises the damage and makes a strand incision adjacent to the dimer^{1–4}. Characterisation of excision-defective mutants of *Escherichia coli* has shown that in this organism early steps of repair are controlled by the *uvrA*, *uvrB* and *uvrC* genes⁵. Although *uvrA* and *uvrB* mutants seem to be incision defective *in vivo*^{6,7}, it has not been possible to measure any difference in the amount of ultraviolet-endonuclease activity between crude extracts from mutants and wild-type cells^{8,9}. After partial purification of wild-type or *uvrC* mutant extracts, however, an ultraviolet endonuclease has

been identified which is absent from *uvrA* and *uvrB* cells⁹ (in this communication termed the *uvrAB* endonuclease). The relevance of these results to whole cells is unclear, because recent experiments with permeable cells have shown that *uvrA*⁺*uvrB*⁺-dependent strand incision requires adenosine-5'-triphosphate (ATP)^{10–12}, whereas the *uvrAB* endonuclease is independent of ATP². The aim of the present investigation was to observe ATP-dependent ultraviolet-endonuclease activity in a cell-free system. We report here the characterisation in crude extracts of an ATP-dependent ultraviolet-endonuclease activity from *E. coli* and conclude that the activity reflects that the enzyme is essential for repair in whole cells. The activity requires the complementary action of the *uvrA*⁺ *uvrB*⁺ and *uvrC*⁺ products and this has been utilised to establish *in vitro* assays for the individual products of these genes.

In preliminary experiments, extracts made by Brij-58 lysis¹³ or lysozyme lysis of freeze-thawed cells¹⁴ were tested for ATP-dependent ultraviolet-endonuclease activity, but results with these extracts were unsatisfactory. Soluble extracts were then prepared by a procedure based on gentle lysozyme lysis of sucrose permeabilised cells (see legend, Fig. 1). This method avoids the use of detergents or freeze-thawing which might affect labile enzyme activities. Typical results obtained by assaying endonuclease activity in extracts from wild-type cells and repair-defective mutants are shown in Fig. 1a–d.

In the absence of ATP, extracts from all strains showed similar levels of ultraviolet-endonuclease activity, in agreement with results reported previously^{8,9}. Addition of ATP however, caused a fivefold increase in the amount of ultraviolet-endonuclease activity in wild-type extracts, but had only a minor effect on mutant extracts. It is thus clear that the major ultraviolet-endonuclease activity in wild-type extracts requires ATP and depends on functional *uvr* genes. The formation of ultraviolet-induced strand incisions in permeabilised cells has the same characteristics^{10,11}, and we infer that the activity observed in these extracts reflects the ultraviolet-endonuclease essential for repair in whole cells.

In view of the common deficiency in extracts from cells with mutations mapping at distinct loci, *in vitro* complementation between different mutant extracts was attempted. When extracts from *uvrC* and either *uvrA* or *uvrB* cells were mixed, the ATP-dependent ultraviolet-endonuclease activity was completely recovered (Fig. 1e–f). Combined extracts from *uvrA* and *uvrB* cells also displayed activity due to complementation, although usually not to the same extent as combinations including *uvrC* extracts (Fig. 1g). Evidently, gene products from *uvrA*⁺, *uvrB*⁺ and *uvrC*⁺ are required for ATP-dependent

Table 1 Effect of various incubation conditions on the ultraviolet-specific endonucleolytic activity from *uvr*⁺ cells

Variation in reaction mixture	Ultraviolet-specific endonuclease activity (%)
Complete reaction mixture	100
–ATP	18
3 mM ATP	115
–KCl	35
3.5 mM Mg ²⁺	38
10 mM Mg ²⁺	100
20 mM Mg ²⁺	75
+25 mM caffeine	10
+10 mM NEM	29

Extracts from cells of strain AB1157 *uvr*⁺ were assayed in duplicate for nonspecific and ultraviolet-specific endonucleolytic activity (DNA exposed to 225 J m^{–2} of ultraviolet). Net ultraviolet-specific activity was calculated subtracting the amount of nicked DNA in non-irradiated samples from the amount of nicked DNA in irradiated samples. The activity obtained in standard assay conditions (Fig. 1) including ATP (1.5 mM) in the reaction mixture was normalised to 100% and the effect of additions (+), omissions (–) or change in concentration of a single component was calculated relative to this value.

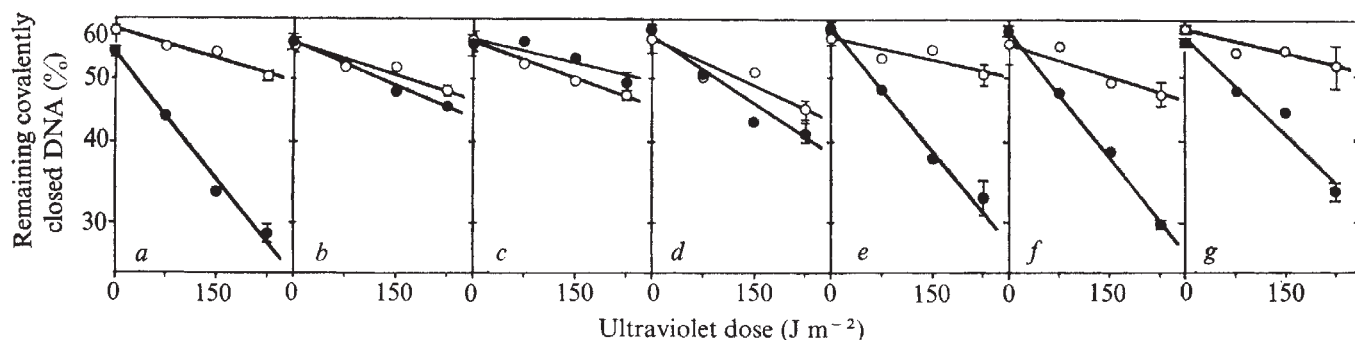


Fig. 1 Ultraviolet-specific ATP-dependent endonucleolytic activity in extracts from excision-proficient and deficient strains of *E. coli* K12. Soluble extracts from strains AB1157 *uvr*⁺ (a), AB1886 *uvrA* (b), AB1885 *uvrB* (c) and AB1884 *uvrC* (d), were assayed for endonuclease activity in the absence (○) or presence (●) of ATP (1.5 mM), using the "nicking" assay of Braun *et al.*⁹ with the modification that covalently closed circular *colE1* DNA (molecular weight = 4.2×10^6) was used as substrate. Plasmid DNA, ³H-labelled, was extracted from growing cells of strain SK2001 *thy*⁻ *colE1* by means of a sodium dodecyl sulphate (SDS)-NaCl lysis procedure, and the covalently closed circular form was purified by phenol extraction and banding in CsCl-ethidium bromide. Details of the purification procedure will be described elsewhere (Nissen-Meyer and Seeberg, to be published). For extract preparation cells were first treated with high sucrose as described by Hurwitz *et al.*¹⁰ and then lysed with lysozyme. Approximately 10^{10} exponentially growing cells were suspended in 0.25 ml 2.5 M sucrose (0.04 M Tris, pH = 8.0, 10 mM EGTA) and the suspension diluted fivefold in a buffer (50 mM MOPS, pH = 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol), containing 125 µg ml⁻¹ of lysozyme. After 45 min on ice, the lysates were centrifuged at 10,000g for 15 min. The supernatant, subsequently referred to as extract, was completely soluble, essentially DNA free (less than 0.1% of total DNA), and contained approximately 6 mg of protein. Standard reaction mixtures (130 µl) contained 40 mM MOPS, pH = 7.5, 85 mM KCl, 15 mM MgSO₄, 1 mM EDTA, 0.8 mM dithiothreitol, 5 ng DNA exposed to various doses of ultraviolet light (254 nm), and 10 µl extract. Incubation was for 15 min at 37 °C. Incised DNA was selectively denatured and separated from intact DNA by filtration through nitrocellulose as previously described⁹. The fraction of remaining covalently closed circles was determined by scintillation counting and plotted against ultraviolet exposure to the DNA. e-g, Results obtained assaying extract mixtures (1:1) of strains AB1886/AB1884 (e), AB1886/AB1884 (f) and AB1886/AB1885 (g). Each value plotted is the average of two or three independent assays and bars indicate standard deviation.

ultraviolet-endonuclease activity and a mutation at any one of these loci does not prevent the formation of functional products from the other *uvr* genes. The complementation provides an *in vitro* assay for the separate *uvrA*⁺, *uvrB*⁺ and *uvrC*⁺ products. The activity of either product can be assayed by *in vitro* complementation with an extract made from the corresponding mutant, measuring ATP-dependent ultraviolet-endonuclease activity. Purification of the *uvrC*⁺ product is now being attempted using this approach. *In vitro* complementation assays have proved very useful in the purification of proteins active in DNA replication^{15,16}.

The ATP-dependent ultraviolet-endonuclease observed in crude extracts resembles the partially purified *uvrAB* endonuclease described previously, in that both activities are stimulated by moderate concentrations of salt and require the gene products from *uvrA*⁺ and *uvrB*⁺ (Table 1, ref. 9). The activity in extracts requires ATP, fairly high concentrations of Mg²⁺ and the *uvrC*⁺ product (Table 1, Fig. 1). In contrast, the *uvrAB* endonuclease is not stimulated by ATP, acts in the absence of Mg²⁺ and is present in normal amounts and with normal activity in *uvrC* cells^{4,9}. These results can be reconciled if the *uvrC*⁺ product interacts with the *uvrAB* endonuclease to yield ATP-dependent ultraviolet-endonuclease activity. The *uvrC*⁺ product may not have a direct role in the production of single-strand breaks, but may possibly be an ATP-requiring factor which promotes the turnover and thereby amplifies the activity of the endonuclease. The activity of the *uvrAB* endonuclease in the absence of the *uvrC*⁺ product may be detectable in extracts from *uvrC* cells where we repeatedly observed slightly more ultraviolet-endonuclease activity than in *uvrA* or *uvrB* extracts (Fig. 1d).

Results with *uvrC* cells *in vivo* have suggested that the *uvrC* function may be involved in a step succeeding incision because single-strand breaks slowly accumulate in the DNA of *uvrC* cells during post-irradiation incubation^{7,17}. The rate of formation of these breaks seems to be increased by a mutation affecting ligase activity, suggesting that the *uvrC*⁺ product may prevent ligase reversal of the incision reaction and force the subsequent removal of the dimer¹⁸. Our results do not eliminate this hypothesis, because ligase may be partially active

in the extracts in spite of the absence of the ligase cofactor nicotinamide adenine dinucleotide in the reaction buffer. It seems clear, however, from the results presented in this communication, that the *uvrC*⁺ product in extracts has an essential role in promoting strand incisions, directly or indirectly. Purification of the different components of the ATP-dependent ultraviolet-endonuclease activity seems necessary before the interrelationship between the *uvrA*⁺, *uvrB*⁺ and *uvrC*⁺ products can be defined in detail.

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THOMAS *et al.*¹ have constructed a derivative of phage λ (λ gt $\cdot\lambda$ C) that is useful for cloning DNA fragments 1,000 to 15,000 base pairs long from other organisms. Their cloning procedure involves cutting the phage DNA into three fragments with *Eco*RI nuclease, removing the central (or "C") fragment, and annealing the outside fragments with an RI digest of foreign DNA. We have recently introduced three amber mutations—*Wam*403, *Eam*1100, and *Sam*100—into λ gt $\cdot\lambda$ C with the object of making the phage less likely to encounter a susceptible host in nature². The NIH Advisory Committee on Recombinant DNA Research, on the basis of appropriate tests, has certified λ gt $WES\cdot\lambda$ C as an EK2 vector^{3,4}.

In order to extend the usefulness of this vector to the cloning of smaller fragments of DNA (such as synthetic, double-stranded DNA derived from the reverse transcription of mRNA) and to obviate the need for biochemically deleting the 5,400 base pairs central *EcoRI* restriction fragment, λ C, we have replaced the λ C fragment with the phenotypically inert *EcoRI* fragment λ B (refs 5-7).

(In addition to genetic tests necessary to verify the mutations present in λ gtWES- λ C, the right and left restriction fragments of the phage must be purified from the λ C fragment before their inclusion in EK2 recombination reactions. The λ C fragment encodes genes involved in integration, *int*, *xis* and *att*, as well as phage-promoted generalised recombination, *red*. This fragment can be accommodated in hybrids with foreign DNA fragments as large as 7,000 base pairs. Thus the λ C fragment must be biochemically removed to minimise the opportunity for recombination between bacterial host DNA and a potentially deleterious hybrid as well as to prevent the outgrowth of *red*⁺ reconstituted parent-like recombinant phage. While this purification can be accomplished by sucrose gradient sedimentation or Agarose gel electrophoresis, both procedures are wasteful of time and materials and may damage the single-stranded portion of the RI cleavage site.)

The new derivative retains all the safety features of λ gtWES- λ C but now lacks the λ C fragment and the λ elements *int*, *xis*, *att* and a portion of the λ *red* gene, which therefore, no longer need to be deleted biochemically. Since the λ B fragment contains two immediately adjacent *Sst*I restriction endonuclease sites (S. Goff, personal communication) which enable λ gtWES- λ B to be cleaved into fragments of sufficient length for packaging, it can also be used in conjunction with various "tailing" and "blunt-end ligation" strategies to clone fragments of DNA less than 1,000 base pairs in length. Here we describe the construction and properties of λ gtWES- λ B.

DNA from the two parental phages, λ gtWES- λ C and λ gt- λ B, were treated with *Eco*RI and permitted to recombine randomly in the presence of T4 DNA ligase (Fig. 1). Plaques obtained after transfection were screened for appropriate markers on three test strains. The desired recombinant contains the left and right arms from the first parent and the λ B fragment from the second parent. The presence of amber mutations was recognised by failure to grow on a *supO* host (*E. coli* 594), and the presence of *Sam*100 by failure to grow on a *supE* host. The red plaque test was used to detect the absence of the middle λ C fragment carrying the *int* and *xis* genes¹³. From candidate recombinants obtained by this screening, the desired phage derivative carrying all three amber mutations was identified by marker rescue studies. Further genetic characterisation has confirmed that this derivative is *red*⁻ and carries *cfts857* and the *nin5* deletion (Table 1).

The selected recombinant, λ gt *Wam403 Eam1100 Sam100*· λ B, was characterised biochemically by its restriction fragment pattern (Fig. 2). *Eco*RI cleaves λ gt *WES*· λ B DNA into the three expected fragments: a 21.3 kilobase (kb) left arm, a 13.9 kb right arm and a 4.69 kb middle fragment characteristic of the λ B fragment obtained from restriction of λ cI857 or λ gt· λ B DNA. The restriction endonuclease *Sst*I cleaves λ DNA at only two adjacent sites within the λ B fragment (S. Goff, personal communication). *Sst*I cleavage of λ gt *WES*· λ B yields the expected three fragments confirming the presence of the λ B fragment. Digestion with *Bam*HI of λ gt *WES*· λ B and λ gt· λ B indicates that a single copy of the λ B fragment has been inserted in the recombinant in reverse orientation. *Bam*HI cleavage of both phage DNAs yields only four fragments. *Bam*HI would generate a fifth easily-distinguishable band if a second copy of the λ B fragment were present, regardless of its orientation with respect to the first. On the basis of their gel mobility, the size of these fragments in λ gt· λ B are 5.4 kb, 16.7 kb, 6.4 kb, and 11.4 kb as would be expected from the λ *Bam*HI cleavage map⁹. However, in the new recombinant the second and third fragments are 19.3 kb and 3.8 kb, respectively, as would be expected from the fragment orientation shown in Fig. 1.

The new vector, λ gtWES- λ B, greatly simplifies the preparation of hybrid recombinants under EK2 conditions. Since the vector lacks the fragment on which genes for integration and

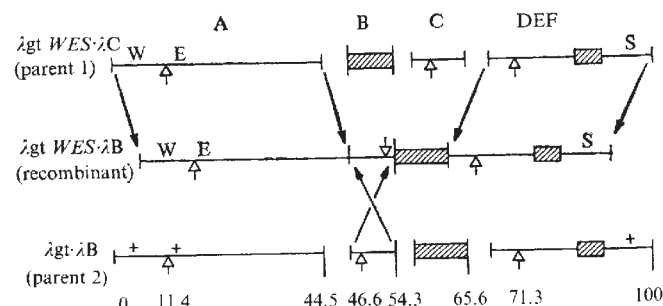


Fig. 1 Construction of λ gt Wam403 Eam1100 Sam100- λ C *Eco*RI fragments of the two parents λ gtWES- λ C and λ gt- λ B are represented as separated solid lines and labelled A, B, C, DEF at the top of the figure. *Bam*HI sites in the phage DNA are represented as arrows and both *Eco*RI (ref. 8) and *Bam*HI (ref. 9) target sites are given at the bottom of the figure as percentage λ unit length. Deletions are represented as hatched boxes. The deletion in the right arm of all three phages is *hins5*. The preparation of λ gt Wam403 Eam1100 Sam100- λ C DNA has been described previously². The construction of λ gt- λ B has been described¹. The phage was prepared from plate stocks¹⁰. λ gt- λ B DNA was prepared from purified phage as previously described². λ gtWES- λ C and λ gt- λ B DNA were digested at 50 μ g ml⁻¹, and 25 μ g ml⁻¹, respectively, by *Eco*RI at 60 U ml⁻¹ in separate 50- μ l reactions containing 0.1 M Tris-HCl, pH 7.9; 50 mM NaCl; 12 mM MgCl₂; 0.1 mM EDTA for 30 min at 37 °C. The reactions were stopped by incubation for 5 min at 70 °C. The DNAs digested with *Eco*RI were then mixed in a 50- μ l reaction mixture containing 20 μ g ml⁻¹ λ gtWES- λ C DNA, 8.3 μ g ml⁻¹ λ gt- λ B DNA, 0.1 M Tris-HCl, pH 7.5; 50 mM NaCl; 12 mM MgCl₂; 0.1 mM EDTA; 10 mM dithiothreitol; 0.1 mM ATP and 50 μ g ml⁻¹ bovine serum albumin and recombined by incubation for 48 h at 9 °C with 0.5 U ml⁻¹ T4 DNA ligase (Miles). The reactions were diluted to 1.0 μ g ml⁻¹ DNA and used to transfect a T1-resistant isolate of JCS183 (*E. coli* F-*shcA5 recB21 recC22 gal- Endo I*) (ref. 11) essentially as described in Cameron *et al.*¹². The transfected cells were plated with the indicator *gal* deletion strain SA825 (*supF*). Plaques which contained amber mutations were scored by failure to grow on *supO* strain 594. Plaques containing Sam100 were scored by poor growth on the *supE* strain C600. Plaques containing the λ B fragment were scored by their formation of white plaques in the test for *int-xis* devised by Enquist and Weisberg¹³. As indicated in Table 1, candidate recombinants were scored for the presence of Eam1100 by their growth on *groE*. The presence of all three ambers was confirmed by marker rescue studies described in Table 1.

Table 1 Genetic analysis of λ vectors

	<i>W</i> *	<i>E</i> *†	(<i>int-xis</i>)‡	Markers assayed <i>red</i> §	<i>clts857</i> ¶	<i>nin5</i>	<i>S</i> *
λ gt- λ C	+	+	+	+	+	+	+
λ gt- λ B	+	+	—	—	+	+	+
λ gt Wam403 Eam1100 Sam100- λ C	—	—	+	+	+	+	—
λ gt Wam403 Eam1100 Sam100- λ B	—	—	—	—	+	+	—

The presence of the genetic marker listed was determined by several methods indicated as follows.

*Complementation tests were performed as described by Shimada *et al.*¹⁴. +, Plaque formation on 594 (*sup0*); —, no plaques observed for the given complementation test.

†Phage carrying the Eam1100 mutation will form plaques (+) on strain Ymel *groE* whereas phage lacking the mutation will not (—) (refs. 2 and 15).

‡The red plaque test for λ *int* and *xis* function was used as described by Enquist and Weisberg¹³. +, Formation of red plaques, thus (*int-xis*)⁺; —, white plaques or (*int-xis*)[—].

§Phage lacking the λ *red* function will not form plaques on *Feb*[—] hosts¹⁶. We used Ymel *ligts7* at 32 °C as the *Feb*[—] host. +, Phage growth on this host; —, no growth.

¶Phages carrying the *clts857* mutation form turbid plaques at 32 °C and clear plaques at 42 °C. +, Phages with this phenotype.

||The presence of the *nin5* deletion was determined as described in Enquist *et al.*². +, Phages carrying the *nin5* deletion.

generalised recombination reside, the larger λ fragments need not be purified and the possibility of low-level λ C fragment contamination is eliminated. As with λ gtWES- λ C, this vector should accommodate DNA fragments as large as 13–15 kb.

The susceptibility of λ gtWES- λ B DNA to *Sst*I nuclease has

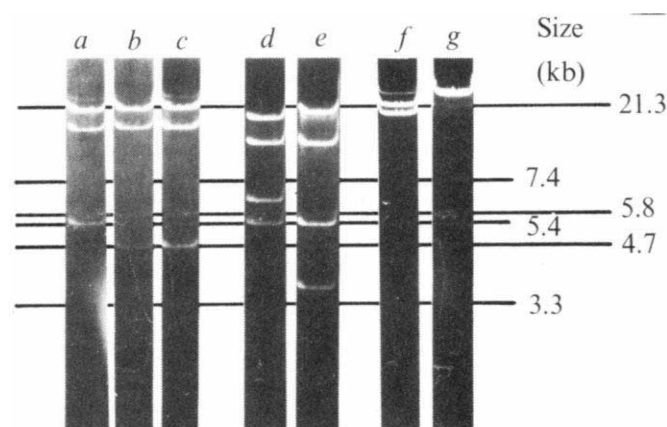


Fig. 2 Restriction enzyme analysis of λ gt Wam403 Eam1100 Sam100- λ B DNA. *a*, λ gt Wam403 Eam1100 Sam100- λ C (*Eco*RI); *b*, λ gt Wam403 Eam1100 Sam100- λ B (*Eco*RI); *c*, λ gt- λ B (*Eco*RI); *d*, λ gt- λ B (*Bam*HI); *e*, λ gt Wam403 Eam1100 Sam100- λ B (*Bam*HI); *f*, λ gt Wam403 Eam1100 Sam100- λ B (*Sst*I); *g*, λ gt Wam403 Eam1100 Sam100- λ C (*Sst*I). The mobility of standard λ cl857 *Eco*RI fragments in this gel system is represented by the horizontal lines and then sizes are given in kilobase pairs. λ gt Wam403 Eam1100 Sam100- λ B phage was prepared by liquid infection initiated at a multiplicity of 0.01 and a cell density of 2×10^8 ml⁻¹. After an 8-h incubation at 37 °C, the suspension was treated with 1% CHCl₃ for 10 min at 37 °C, brought to 10 mM MgCl₂, and centrifuged for 10 min at 20,000*g* to remove cell debris. λ cl857, λ gt Wam403 Eam1100 Sam100- λ C and λ gt- λ B phage were prepared from induced lysogens or plate stocks¹⁰. Phage purification and DNA isolation have been previously described². DNA solutions (30 to 830 μ g ml⁻¹) are stored at 0–5 °C in 10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA. *Eco*RI was purified from *E. coli* BRY 13.3/1100.5 (to be described elsewhere). Purified *Sst*I was the gift of Dr Steven Goff. *Bam*HI was obtained from Bethesda Research Labs. The standard restriction reaction contained 0.3 μ g DNA in 25 μ l and sufficient enzyme to cleave 1.0 μ g DNA. The *Eco*RI buffer was 0.1 M Tris-HCl, pH 7.9; 50 mM NaCl; 0.1 mM EDTA; 12 mM MgCl₂. The *Sst*I and *Bam*HI buffer was 20 mM Tris-HCl, pH 7.5; 7.0 mM MgCl₂; 60 mM NaCl; 2 mM β -mercaptoethanol. The reactions were incubated for 45 min at 37 °C. After subsequent incubation for 5 min at 75 °C, the reactions were electrophoresed through 1.0% Agarose (ScaKem) slab gels as described previously¹⁷. The DNA was stained by immersion in 0.0025% ethidium bromide for 3 min and made visible under an ultraviolet lamp. The small 1.0-kb band generated by the *Sst*I cleavage is not readily visible in the contact print of this gel system.

two implications. First, it should be possible to treat the vector with *Sst*I as well as *Eco*RI before hybrid formation to eliminate the bulk of parental recombinants. The *Eco*RI λ B fragment when reduced to two fragments each with one *Eco*RI and one *Sst*I end will still anneal to other *Eco*RI-ended fragments but will require the ligation of two *Eco*RI cleavage sites and an *Sst*I cleavage site in order to form a viable transfectant. The introduction of foreign DNA fragments containing only *Eco*RI ends requires only the annealing and ligation of two *Eco*RI sites. Second, the two arms created by *Sst*I cleavage of λ gtWES- λ B DNA are sufficiently longer than the *Eco*RI arms to allow them to be resealed into plaque-forming DNA. *Sst*I arms thus represent a potential EK2 vector for insertion of low molecular weight double-stranded DNA molecules which are too short for insertion into the *Eco*RI arms alone.

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RNA pattern of 'swine' influenza virus isolated from man is similar to those of other swine influenza viruses

IN February 1976, during an epidemic of influenza among military recruits at Fort Dix, New Jersey, five virus isolates were obtained which contained haemagglutinin and neuraminidase antigens indistinguishable from those of isolates of swine influenza virus¹. Subsequent evidence of elevated serum antibody titre against 'swine' influenza virus in hundreds of individuals at Fort Dix² prompted the US government to launch a national immunisation programme against 'swine' influenza virus. One current theory suggests that new, pandemic strains of influenza virus result from genetic recombination of human influenza A viruses and strains of virus that are resident in animal populations^{3,4}. According to this hypothesis, recombinants may emerge containing genes derived from the animal virus which code for novel surface proteins, and genes derived from the human virus which confer properties of virulence in man. We have demonstrated that the genetic composition of recombinant viruses could be determined by comparison of RNA patterns of recombinant and parent viruses on urea-polyacrylamide gels⁵⁻⁷. We have therefore used the same techniques to explore the possibility that the 'swine' virus isolated from recruits at Fort Dix is a recombinant of swine influenza virus from pigs and H3N2 influenza A virus currently prevalent in man.

Figure 1 shows the RNA patterns of three low passage isolates of 'swine' virus obtained from three patients at Fort Dix. All three isolates are identical with respect to the migration of the eight corresponding RNA segments on urea-polyacrylamide gels, confirming previous observations regarding the reproducibility of the RNA patterns in this system.

We compared the RNA pattern of the New Jersey 'swine' virus isolate with those of four influenza viruses isolated from swine and with that of a H3N2 virus isolated from recruits during the same epidemic at Fort Dix in February, 1976. Figure 2 demonstrates the results of one experiment, but it should be noted that at least three different ³²P-labelled RNA preparations were made from each virus and that numerous gels were run to verify the results.

Figure 2 shows that the RNA pattern of the New Jersey 'swine' virus, A/NJ/11/76, isolated from man (lane 2) is virtually identical to that of the swine virus isolate A/swine/Taiwan/1/75 (lane 3), and except for the fifth RNA segment closely resembles the pattern produced by another swine virus isolate, A/swine/Tennessee/1/75 (lane 4). Comparison of a third swine virus, A/swine/Wisconsin/1/73 (lane 7), and the New Jersey 'swine' virus (lane 8) reveals close similarity of the top four RNA segments as well as slight differences in mobility of RNA segments five to eight. In contrast, each of the RNA segments of the H3N2 virus, A/NJ/743/76 isolated from recruits during the same epidemic at Fort Dix (lane 1) could be distinguished from the corresponding RNA segments of the New Jersey 'swine' virus (lane 2), except possibly for RNA segment three. Note that the fourth segment of both viruses codes for haemagglutinin and the fifth segment of A/NJ/743/76 (H3N2) and the sixth of A/NJ/11/76 (Hsw1N1) code for the respective neuraminidases^{7,8}. In particular, swine viruses were characterised by a broad diffuse band at the top in which RNAs 1 and 2 cannot easily be resolved. Furthermore, the eighth RNA segments of all swine viruses migrate faster than the corresponding segments of H3N2 viruses.

Figure 2 also demonstrates the RNA pattern of an H3N2 virus isolated from swine in 1970, A/swine/Taiwan/1/70 (lane 6), which except for RNA segment seven is clearly distinguishable from patterns produced by the other swine virus isolates. This observation is in accord with the previous assumption that this strain, A/swine/Taiwan/1/70, is a virus of man which crossed

species barriers and infected pigs¹⁰. (Comparison of the RNAs of this virus with those of A/Hong Kong/8/68 virus on other gels reveal very similar patterns.)

Figure 3 is a photograph of another gel demonstrating the RNA patterns of some of the viruses not completely resolved

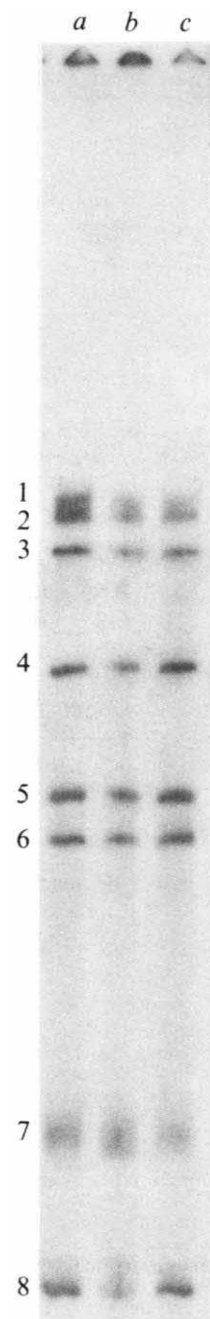


Fig. 1 Analysis of RNAs of three New Jersey 'swine' influenza virus isolates on a 6 M urea-polyacrylamide gel. RNAs were labelled with ³²P during a one-step growth cycle in MDCK (canine kidney) cells as described previously⁵⁻⁷. Electrophoresis on a 6 M urea (2.6%)—polyacrylamide gel was carried out according to previously published procedures⁵⁻⁷. Migration was from top to bottom. The RNA segments are numbered 1 to 8. *a*, RNA of influenza A/NJ/11/76 (New Jersey 'swine'; Hsw1N1) virus; *b*, RNA of influenza A/NJ/8/76 (New Jersey 'swine'; Hsw1N1) virus; *c*, RNA of influenza A/NJ/9/76 (New Jersey 'swine'; Hsw1N1) virus. Throughout the manuscript we are using the system of nomenclature recommended by the WHO⁹. According to this system only viruses isolated from swine carry the strain designation swine. The strains isolated from recruits at Fort Dix, which are characterised by swine virus surface antigens, are, however, referred to as New Jersey 'swine' viruses in this report.

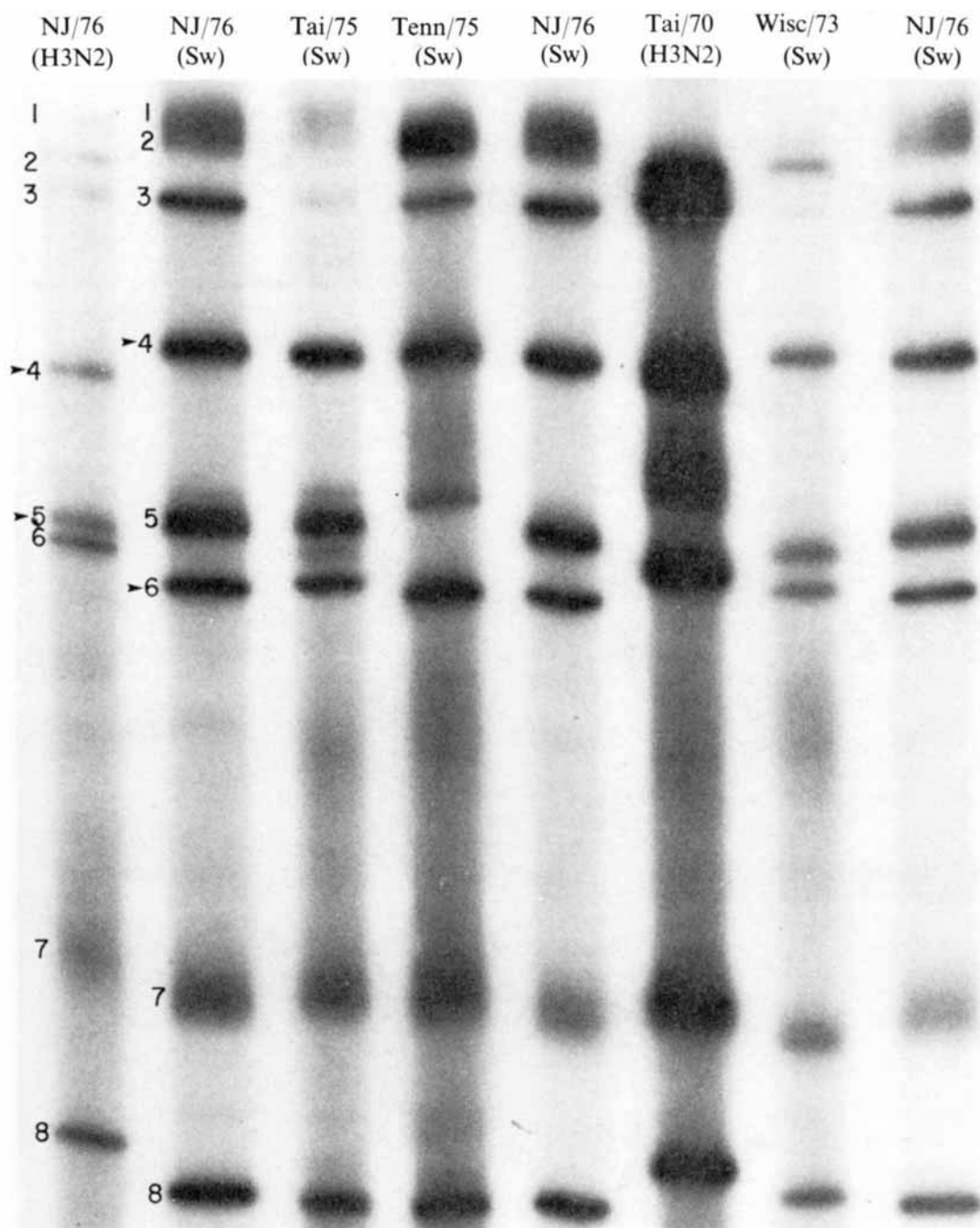


Fig. 2 Comparison of the RNA pattern of the New Jersey 'swine' influenza virus with those of A/NJ/743/76 (H3N2) virus and four viruses isolated from pigs. Conditions of electrophoresis on the 6-M urea-polyacrylamide gel are as in Fig. 1. The RNA segments of A/NJ/743/76 and the New Jersey 'swine' virus (A/NJ/11/76) are numbered 1 to 8. The haemagglutinin genes (RNA 4) and the neuraminidase genes (RNA 5 or 6) of these two viruses are identified by arrows^{7,8}. Lanes: 1, RNA of influenza A/NJ/743/76 (H3N2) virus; 2, 5 and 8, RNA of A/NJ/11/76 (New Jersey 'swine'; Hsw1N1) virus; 3, RNA of influenza A/swine/Taiwan/1/75 (Hsw1N1) virus; 4, RNA of influenza A/swine/Tennessee/1/75 (Hsw1N1) virus; 6, RNA of influenza A/swine/Taiwan/1/70 (H3N2) virus; 7, RNA of influenza A/swine/Wisconsin/1/73 (Hsw1N1) virus; note that in the conditions of labelling and growth used, virus with equimolar distribution of RNAs is not always obtained.

in Fig. 2. (We found it difficult to obtain clear resolution of each sample when many viruses are compared on a single gel.) Again it can be seen that the pattern of the New Jersey 'swine' virus in lane 2 resembles that of A/swine/Wisconsin/1/73 (lane 3) and can be distinguished from that produced by either the 1976 H3N2 strain, A/NJ/743/76, (lane 1) or the H3N2 strain, A/swine/Taiwan/1/70, isolated from swine in 1970 (lane 4).

Based on this preliminary analysis, we suggest that the New Jersey 'swine' strain is closely related to other swine viruses—particularly the A/swine/Taiwan/1/75 virus—with respect to all of its 8 genes and most likely was not derived by recombination involving the current human H3N2 strain.

We recognise potential pitfalls in this kind of RNA analysis. First, slight differences in migration rates of comparable RNA segments of two viruses may reflect only minor differences in nucleotide sequences, masking genetic homology. Second, it is possible that the New Jersey 'swine' virus isolate is a recombinant which derives some of its genes from an unrecognised H3N2 parent. Although we have compared the RNAs of five different H3N2 strains with that of the New Jersey 'swine' virus we are aware that these represent only a small sample of potential parents. In addition, we cannot exclude the fact that some small differences in RNAs may not be detectable by this method. Consequently a definitive analysis of the genetic composition

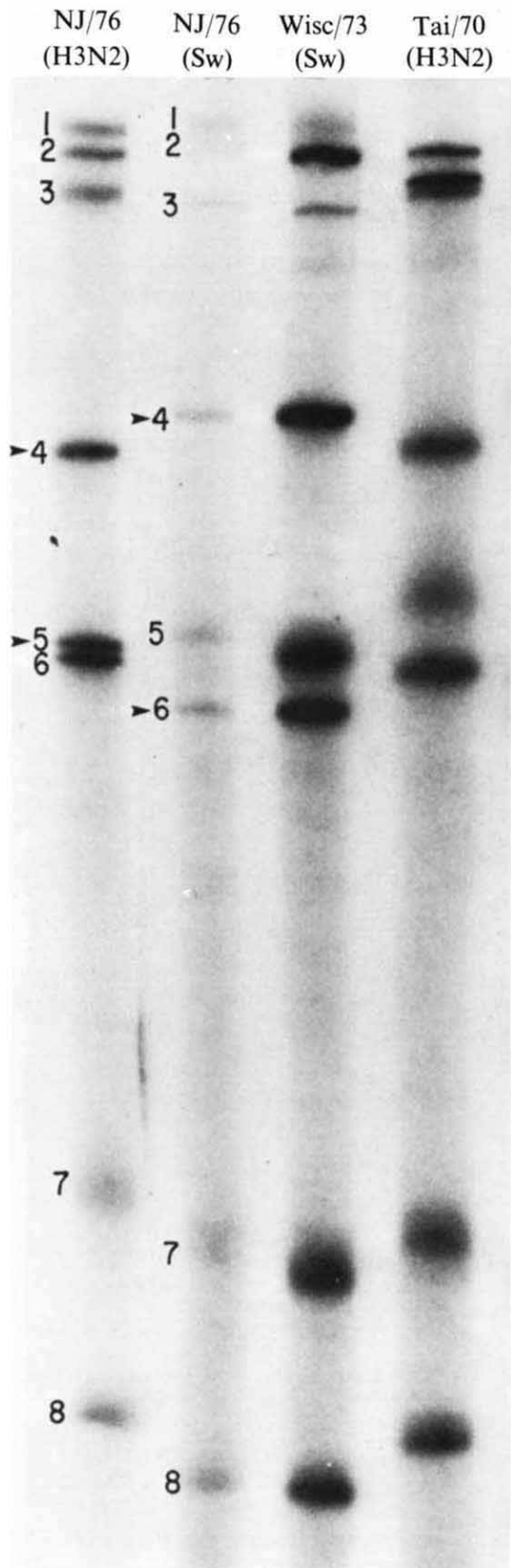


Fig. 3 Analysis of the RNAs of the New Jersey 'swine' influenza virus with those of A/NJ/743/76 (H3N2) virus and two isolates from pigs. Conditions of electrophoresis are as in Fig. 2 but new RNA preparations were used. The RNA segments of A/NJ/743/76 and the New Jersey 'swine' virus (A/NJ/11/76) are numbered 1 to 8. Arrows identify the haemagglutinin genes (RNA 4) and the neuraminidase genes (RNA 5 or 6)^{7,8}. Lanes: 1, RNA of influenza A/NJ/743/76 (H3N2) virus; 2, RNA of influenza A/NJ/11/76 (New Jersey 'swine'; Hsw1N1) virus; 3, RNA of influenza A/swine/Wisconsin/1/73 (Hsw1N1) virus; 4, RNA of influenza A/swine/Taiwan/1/70 (Hsw1N1) virus.

of the New Jersey 'swine' virus can only be obtained by more sensitive and elaborate techniques such as oligonucleotide fingerprinting of all of the RNAs or peptide mapping of each of the gene products.

It is conceivable that the New Jersey 'swine' virus acquired properties of virulence in man after selection during sequential passage in man; but if recombination between human and animal strains is generally required for the emergence of new strains virulent for man, we would regard the New Jersey 'swine' virus to be an unlikely candidate for the next influenza pandemic. (Since the submission of this manuscript it has been reported that the New Jersey 'swine' virus is in fact less virulent for man than other human influenza virus isolates¹¹.)

We thank Ms Marlene Lin and Ms Kaye Leitzinger for technical assistance, and Drs W. R. Dowdle, B. Easterday, M. Goldfield and R. G. Webster for viruses. This work was supported by the NIH and the NSF.

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Errata

In the article "Three step yielding of load clamped mammalian cardiac muscle" by P. Housmans and D. L. Brutsaert (*Nature*, 262, 56; 1976) line 30 on page 57, . . . length change, of phase 2 and the magnitude and speed of . . . should be replaced by . . . the clamp (Fig. 2, lower), disclosed that the length change of . . .

In the article "The zoo: 150 years of research" by L. Harrison Matthews (*Nature*, 261, 281; 1976) the illustration at the top of page 282 is of the walrus *Trichechus rosmarus* and not the bearded seal as indicated.

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matters arising

Bicuculline and visual responses

DUFFY *et al.*¹ proposed that the influence of intravenously administered bicuculline on the receptive field properties of neurones in the visual cortices of cats which have experienced monocular deprivation indicates the "active inhibition of the relatively intact input from the amblyopic eye" by information arising from the normal eye.

In investigations of this type, it hardly seems necessary to expose unanaesthetised, paralysed animals (admittedly locally anaesthetised "to avoid possible confounding" of the results) to bicuculline administered systemically, particularly when its action "was often complicated by its potent convulsive effects". The microelectrophoretic administration of bicuculline or other GABA antagonists near physiologically identified neurones in various regions of the visual system would be likely to provide more definitive evidence regarding the involvement and localisation of GABA-mediated inhibitory mechanisms in binocular vision, and their perturbation as a consequence of monocular deprivation, with considerably less distress to the experimental animal.

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¹ Duffy, F. H., Snodgrass, S. R., Burchfiel, J. L., and Conway, J. L., *Nature*, 260, 256-257 (1976).

DUFFY ET AL. REPLY—Curtis has raised¹ at least two issues. He seems to imply that the use of locally anaesthetised animals is improper or unwarranted and, second, he raises the issue of the proper place of iontophoresis in pharmacological studies of the amblyopic animal.

We feel that experimenters have an obligation to minimise an animal's discomfort. No procedure, however, can successfully claim total freedom from trauma. General anaesthesia is probably the best in this regard and should be used whenever possible. General anaesthetics do act on the brain and, in our experience, modify the receptive field characteristics in the visual and somatosensory system. Furthermore,

anaesthetic agents may interact with other experimentally administered drugs, thereby confounding experimental findings and possibly requiring additional experimentation. Since most neurophysiological and neuropharmacological investigations result in an animal's death, we feel that experimenters have an equal obligation to minimise the number of animals used.

In the course of behavioural studies of amblyopic cats, we have administered bicuculline to a number of awake and unrestrained animals. Bicuculline seems to have a sedative effect at low and medium dosage levels. At higher levels, epileptic activity in the EEG and convulsions occur quite suddenly and result in an immediate loss of consciousness. Cats do not seem unduly distressed at any time. For all these considerations, we felt that the locally anaesthetised preparation was best for our initial experimentation.

We agree with Curtis that iontophoresis is especially useful for discerning the location of a given drug effect within the nervous system, but feel that it has some problems as an exploratory technique. For example, a negative iontophoretic study with bicuculline in amblyopic cats would not have great meaning unless many regions were sampled which would necessitate the use of more animals. We would also point out that when the data obtained with iontophoretic and intravenous drug administration are in apparent conflict, it is by no means certain that the iontophoretic data give a correct perspective. For example, we cite the recent work of Ben-Ari and Kelly² where iontophoretic, but not intravenous, flupenthixol blocked the response to iontophoretically applied dopamine. We do plan to make use of iontophoresis in the future and expect that it will be useful in elucidating the detailed mechanism of effect.

Finally we agree that intravenous bicuculline is a toxic agent and have been seeking a safer agent with a longer duration of action. We believe that intravenous administration of ammonium salts may meet these requirements.

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¹ Curtis, D. R., *Nature*, 263, 531 (1976).

² Ben-Ari, X., and Kelly, X., *J. Physiol., Lond.*, 256, 1 (1976).

Mammalian cell growth regulation

HOLLEY has proposed that mammalian cell growth regulation in culture is regulated primarily by the depletion of diffusible resources, especially polypeptide factors; that the responsiveness of cells to such factors is density dependent; and that this responsiveness changes in a characteristic manner following transformation.

If this theory is correct, the following must be true: (1) that normal and malignant cells differ demonstrably in their growth regulatory policies; (2) that growth control involves a conventional density dependence; (3) that growth inhibition at high density is not merely the result of culture starvation by careless investigators; and (4) that mechanisms other than diffusible substances contribute little to growth regulation.

It may fairly be stated that there is no *in vitro* cell characteristic which has ever been rigorously demonstrated to correlate with and be diagnostic of the malignant state for a broad spectrum of cell types. The following *in vitro* indices are not broad spectrum malignancy correlates: saturation density, multilayering, contact growth inhibition, growth rate, and growth in low serum²⁻¹⁰; cell adhesiveness and surface charge¹¹⁻¹⁴; lectin agglutinability¹⁵⁻¹⁸; fibrinolytic activity^{19,20}; cell morphology and karyotype^{8,9,21,22}. A recalculation of chi-square values for the data of ref. 23, assuming 3T3 cells to be malignant because of their production of tumour angiogenesis factor²⁴ and *in vivo* tumorigenicity², casts grave doubt on the lack of anchorage dependence as a valid tumorigenicity index. Similarly, the status of the LETS-SF complex is in doubt because many studies of it have used as "normal" controls either cell lines of untested tumorigenicity or lines known (3T3 (ref. 2), BHK21 (refs 9 and 25) or suspected (Wi38 ref. 24)) to be tumorigenic. Holley's argument about growth policy differences between normal and malignant cells is clearly without foundation.

Holley contends that density-dependent growth regulation is "due to a quantitative increase in the requirements for macromolecular growth factors as cell density increases." His only basis for this statement is the citation of three references which speculate about growth regulation by the density-dependent

control of cell surface area and nutrient uptake, a theory which now seems unlikely^{34,35}, and "wound healing" in monolayer culture. This wound healing, once thought to be density dependent, now seems to involve the destruction of an external diffusion barrier near the cell surface³⁶. In certain culture systems, growth inhibition definitely does not involve density dependence³⁷.

Holley defines density-dependent growth regulation as a tendency for cells "to grow to a 'saturation' density and then stop growing." This definition is unsatisfactory. Density dependence is a change per cell in the value of a cellular property as population density changes. As a rule, a density dependence of growth operates at all population densities and may involve either or both the promotion and inhibition of growth rate^{10,26,27}. In culture, the inhibition of cell growth usually occurs even when nutrients and diffusible factors are in excess and not rate limiting^{3-5,10,27-33}. Although in well fed cultures growth inhibition is occasionally observed⁴, it occurs at much higher densities than are normally employed and probably reflects the restriction of diffusional transport either by an external diffusion barrier³⁶ or by multilayering. Growth inhibition and its density dependence in culture are not usually the result of resource depletion except for the special situation in which the investigator fails to provide adequate nutrient to his cultures. In general, both 'saturation' density and quiescence are starvation artefacts^{3-6,10,27-33}.

Holley's acceptance of the physiological significance of certain diffusible growth effectors is uncritical. Of the materials he cites, only the nerve and epidermal growth factors (NGF, EGF) are clearly growth regulators *in vivo*. Plant lectins certainly are not, cyclic AMP probably is not³⁷⁻⁴⁰, while the role of fibroblast growth factor, insulin, hydrocortisone, prostaglandins, antigens, and proteolytic enzymes *in vivo* is unknown. Since blood vessels are selectively permeable, the mere presence of a growth effector in serum does not mean that the substance regulates cell growth. Its presence in the interstitial fluid surrounding a suspected target cell must also be demonstrated. Similarly, a growth effector from one species of vertebrate cannot be considered physiological when applied to cells from another, as is the case with several of the studies Holley cites. Even Holley's use of the term 'polypeptide factors' is in error. Such factors (S₂, erythropoietin, EGF, NGF, S₁) are often multimers and often of very high molecular weight (26, 46, 74, 140, 600 kdalton respectively); or very small and not polypeptides at all (putrescine, uridine, adenine).

Finally, recent evidence raises the possibility that the extracellular matrix

Matters arising

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may contribute to growth regulation in some systems⁴¹⁻⁴³, and that contact interactions may also²⁷, though not in the manner postulated by Todaro and others.

In summary, growth regulation is complex, confusing, and not at all understood. Certain diffusible substances (NGF, EGF, erythropoietin, colony-stimulating activity) are clearly physiological growth regulators. And certainly density-dependent growth regulation occurs in many culture systems, though it is far more complex and often differs fundamentally from Holley's description of it. While density-dependent growth regulation is sometimes mediated by diffusible substances, it is probably at times mediated by matrix and contact interactions as well. There is at present no evidence for density-dependent increases by cells in their resource requirements, nor is there evidence that resource depletion regulates growth *in vivo*. Even in culture, resource depletion seldom contributes to growth regulation of well fed cells. Finally, no general difference between normal and malignant cell growth regulation in culture has ever been rigorously proved.

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HOLLEY REPLIES—Growth regulation is complex, but I believe Skehan makes the subject unnecessarily confusing.

It is unlikely that all tumors are identical, so it is unrealistic to expect the absolute correlation of tumorigenicity with a single property as Skehan demands¹. Nevertheless, there are differences between "normal" and tumorigenic cells and the two types of cells often differ greatly in their growth behaviour in cell culture. It is important to understand the differences that are observed and this was the subject of the review².

Skehan is influenced by what he considers to be a discovery of "growth inhibition" at low cell density³. In my view, Skehan has not discovered a growth inhibition but rather uses the term incorrectly, and has confused the literature. Cell cultures that begin with quiescent cells normally show a lag period, then a period in which there is one relatively synchronous cell division, and then an extended period of asynchronous growth. Skehan plots³ such a growth curve in the form of growth rate per day and concludes that there is "growth inhibition" at the end of the period of synchronous cell division, since the rate of appearance of new cells falls. In my view, the transition from a brief period of synchronous cell division to asynchrony in a growing population is not properly called "growth inhibition."

There are several inaccuracies in Skehan's present comments¹ but they will be detected by anyone who reads the review².

The Salk Institute

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reviews

Credentials of astro-archeology

A. J. Meadows

Megaliths, Myths and Men: An Introduction to Astro-Archaeology. By Peter Lancaster-Brown. Pp. 324. (Blandford: Poole, Dorset, 1976.) £4.75.

Is there such a subject as astro-archaeology? The word obviously implies not only that pre-literate cultures possessed an astronomical tradition, but also that some part of the tradition can be retrieved from surviving artefacts. The most frequently cited instance of such retrieval—and the one that forms the theme of this book—is provided by the megaliths and their alleged orientation towards astronomically significant points on the horizon. The problem is the degree of certainty that can be attached to any conclusions—after all, some of the proposed orientations, with their accompanying interpretations, are still in dispute today after nearly a century of discussion.

A central difficulty is that we are not dealing with a single debate, but with a range of topics not all of which are contested with equal vigour. At one extreme, there is the basic question—Do any astronomically significant orientations of megaliths indisputably exist? Then, somewhere in the middle, one might ask whether astronomically sophisticated orientations—for example, to particular bright stars—occur. At the other end of the spectrum, can any significance at all be attached to the so-called 'leys'—that is, alleged alignments of widely differing prehistoric features, often over considerable distances?

Mr Lancaster-Brown's book, which examines all these questions, is intended for the uninitiated. Technical astronomy is not avoided, but the necessary concepts and jargon are introduced as the need arises. A genuine beginner may find the problems of the celestial sphere occasionally hard to visualise, but the explanations are generally clear. The text is primarily concerned with present-day attitudes, but we are also given an extended survey of the historical development of interest in the subject. These two themes are by no means distinct, for part of the continuing controversy over the credentials of astro-archaeology has derived from the excessive claims of some of its past adherents. A short diversion outside

Western Europe to examine pyramidology ties in both with the main discussion of astronomically oriented monuments, and with a subsidiary question that has often been raised simultaneously: whether or not the builders of megalithic structures used a standard measure of length.

In terms of our initial range of topics, as Mr Lancaster-Brown notes, the majority of people who have examined megaliths now believe that astronomical orientation of some sort is certainly present. At the other extreme, few—except occultists—give much credence to leys. But the uncertain area remains the exact level of astronomical sophistication enshrined in the megaliths. Quite clearly, opinion in recent years has tended to allow our distant ancestors a more detailed acquaintance with astronomy than hitherto. This change has been partly

caused by the accumulation of data from new and more detailed surveys; but partly it stems from a somewhat greater willingness on the part of archaeologists to accept that our ancestors were actually capable of such sophistication. It is unfortunate in this respect that so much space in the book is devoted to Stonehenge, since, although it may be fascinating from the point of view of speculative astronomy, it is one of the astronomically over-explained monuments in the eyes of archaeologists. Again, archaeologists might wish to quibble at one or two omissions from the list of select references. But the book adequately fulfills its role as an introductory survey. □

Professor Meadows is Head of the Department of Astronomy and History of Science at the University of Leicester, UK.

Soil science and archeology

Soil Science and Archaeology. By Susan Limbrey. Pp. 384. (Academic: London.) £8.40.

In recent years there has been a marked increase in the application of science to archaeology, but soil science has tended to be under-emphasised; this book corrects such a deficiency. The first part of the book summarises the nature of soil properties and pedogenesis. These principles are demonstrated in the following two parts by the description and evolutionary analysis of soils from selected environments. In the fourth part attention is focused on the archaeological attributes of soils.

The inevitable problem with a book of this title is defining the audience to whom it is addressed; it could have been written as a research text for soil scientists involved with archaeology or as an introductory text on soils relevant to field archaeologists. Instead it is written for archaeologists at all levels, but many will find the first part hard going, since a firm scientific background is assumed. Indeed the first three parts will prove to be of great use to students of soil science *per se*; in

particular, the chapters on the history of podzolised and chalk soils in Britain are scholarly articles in their own right.

The only general criticism which can be made is that the reader will not gain detailed advice on how to carry out various procedures—for example, the classification and mapping of soils. A common request to a soil scientist on an archaeological project is a soil or terrain map, and the fieldwork has usually to be completed within a short field season. A demonstration of the necessary techniques could have centred around the greater use of examples and illustrations.

But Dr Limbrey has produced an authoritative text which deserves a wider audience than its title might imply. The usefulness of soil science to archaeology is very well demonstrated, and the potential contribution of archaeology to our understanding of soil evolution is equally stressed.

Donald A. Davidson

Donald Davidson is a lecturer in the Department of Geography at the University of Strathclyde, UK.

Enzyme electrophoresis

Handbook of Enzyme Electrophoresis in Human Genetics. By Harry Harris and D. A. Hopkinson. (North-Holland: Amsterdam, 1976.)

RECOGNITION that enzymes may occur in multiple, electrophoretically distinct, forms first found important practical applications in clinical laboratories where attention has been concentrated on the relatively few isoenzymes known to be of diagnostic significance. The discovery of isoenzymes, however, has had an even greater impact in genetics. Enzyme heterogeneity has been studied by electrophoretic and other techniques at the MRC Human Biochemical Genetics Unit for about two decades. The authors have now provided a distillate of their vast experience in this handbook which is supplied as loose leaves in a ring binder to which supplements can be added as they become available.

After briefly outlining the significance of isoenzymes, the principles of enzyme electrophoresis and methods for their subsequent detection, techniques for the more important methods, especially starch-gel electrophoresis, are described in sufficient detail to enable them to be set up elsewhere. The main part of the work is devoted to individual enzymes with summaries of necessary technical modifications and examples of the patterns obtained. Finally, brief tabulated sections deal with enzyme subunits and the effects of thiol reagents, together with useful appendices.

As would be expected from such distinguished authors, the work is authoritative and well written. The methods are clearly and concisely presented and, although no attempt is made to give a complete bibliography, sufficient key references are included to enable original sources to be traced. Minor criticisms concern the use of certain obsolescent terminology—for example, glutamate-oxaloacetate transaminase—and the lack of standardisation of the abbreviations. These, however, are trivial matters which do not detract from the value of the book. It will be useful, not only to geneticists, but also to clinical biochemists who at any time may encounter hitherto unsuspected enzyme anomalies.

J. H. Wilkinson

J. H. Wilkinson is Professor of Chemical Pathology at Charing Cross Hospital Medical School, University of London, UK.

Subnuclear components

Subnuclear Components: Preparation and Fractionation. Edited by G. D. Birnie. Pp. 334. (Butterworth: London and Boston, Massachusetts, March 1976.) £15.

Subnuclear Components is a valuable book for the practising research worker. It explains the immediate reasoning behind some popular preparative techniques in an area in which the end justified the means. Happily, assessment of the end-product is included in most of the chapters. The discussions all lie in the areas between detailed descriptions of techniques and expositions of general principles, and provide a very valuable insight into the thinking and background associated with some important experimental procedures. Each of the eight chapters is written by a different author or group of authors, all of whom are busy research workers themselves; and it is not surprising therefore that the text and proof reading are not as polished as I would

otherwise expect for £15 per copy. Less excusable is the long gap between writing and publication, during which time some material has been left behind by events such as the nucleosome model for chromatin; and quite crazy is the tiny print used for the figure captions. Although many of the main techniques have been covered there are gaps in such areas as ribonucleoprotein particles, mitotic apparatus and DNA fractionation (DNA preparation is well covered in a chapter by Dr Butterworth). In the best chapters the available methods are critically discussed and a few chosen for detailed analysis. There are, however, some important gaps—for example, in the otherwise excellent discussion of histone preparation, chromatographic methods are almost ignored. Such problems are inevitable when the authors are presenting the detailed 'behind-the-scenes' analysis which make *Subnuclear Components* so valuable. **Harry R. Matthews**

Harry R. Matthews is a senior lecturer in the Department of Physics at the Portsmouth Polytechnic, UK

Protoplasmic connections in plants

Intercellular Communication in Plants: Studies on Plasmodesmata. Edited by B. E. S. Gunning and A. W. Robards. Pp. xvi+387. (Springer: Berlin and New York, 1976.) DM72; \$29.60.

THIS volume contains 14 review papers presented and discussed at a meeting held in Canberra in June 1975. The aim of the volume according to the editors is to summarise what is known about the nature of plasmodesmata and assess the significance of these intercellular protoplasmic connections in plant physiology. The contributors have performed a valuable service in collecting together information which was previously scattered widely in the literature, and the editors are to be congratulated on the skill with which they have integrated the papers and summarised the discussions at what must have been a very lively meeting.

It is now nearly 100 years since Tangl described strands of protoplasm traversing the cell walls in the endosperm of certain seeds, but because of their extreme fineness (30–60 nm) their very existence remained in some doubt until the advent of the electron microscope. As Robards points out in this volume, in spite of intensive study in the past 10 years, many of the details of struc-

ture remain unresolved. In particular the relationship between the desmotubule and the endoplasmic reticulum of the adjoining cells is still uncertain.

Even greater controversy surrounds the operation of plasmodesmata and much of the volume is devoted to this problem. The early suggestions of Haberlandt that protoplasmic connections may function both in the translocation of substances and transmission of stimuli are supported by a wealth of more recent circumstantial evidence, but an unequivocal demonstration that things actually move through plasmodesmata is still awaited. Tyree has established that plasmodesmata may have sufficient capacity to transport solutes at the fluxes observed, but the basis of such transport is still obscure. This volume, with its extensive bibliography, will form an excellent springboard for anyone wishing to advance our knowledge in an intriguing field of research.

The book is printed from 'camera-ready' typescripts without justified margins and one might have expected that the saving in time and cost would be reflected in more rapid publication and lower price than has been achieved.

James F. Sutcliffe

James Sutcliffe is Professor of Plant Physiology in the School of Biological Sciences at the University of Sussex, UK, and Editor-in-Chief of Annals of Botany.

obituary

John Richardson Marrack, DSO, MC, Emeritus Professor of Chemical Pathology in the University of London, died in the USA on June 13, 1976. He was born on November 26, 1886 at Clevedon, Somerset, but soon moved to Tiverton, where he attended Blundell's School, to which he remained greatly attached, and of whose Old Boy's association he later became Vice-President. He went to St John's College, Cambridge and then to the London Hospital Medical College, graduating in 1908.

His first research was on rheumatoid arthritis as a John Lucas Walker student, and later Beit Memorial Fellow at the laboratories of the Cambridge Research Hospital (which has become the Strangeways Laboratory). After the First World War, when he served in the RAMC, he went to the London Hospital as lecturer in Chemical Pathology. He became interested in the properties of colloids, initially from studying the binding of calcium by serum proteins, and came to the conclusion that colloid interactions were caused by definable and verifiable physical and chemical forces, acting between distinct protein entities. As an example he chose antibodies, whose nature was quite unknown and whose very existence as separate entities was doubted. In 1930 he showed that diphtheria antitoxin behaved as a distinct protein whose interaction with diphtheria toxin could be measured quantitatively. In a monograph published in 1934 (*The Chemistry of Antigens and Antibodies*) he proposed that the specific affinity of antibodies for antigens is determined by the same factors which determine the specific binding of molecules to form crystals, that is, the shape of the molecules and the spatial distribution and strength of

polar forces. The monograph contains a clear diagram elaborating the theoretical studies of Heidelberger and Kendall to illustrate what has now become accepted as the 'lattice hypothesis' of antigen-antibody interactions.

Revised in 1938 this work has had a lasting influence, and convinced many chemists and biochemists that immunology was a fit subject for scientific study by themselves as well as by bacteriologists and serologists. Marrack was also the first to use methods which are now commonplace: equilibrium dialysis, whereby he indicated that anti-hapten antibodies were probably bivalent, and the attachment of coloured dyes to antibacterial antibodies, which inspired Albert Coons later to develop the technique of immunofluorescence. Marrack wrote few papers, by present day standards, and his encyclopaedic knowledge of immunochemistry appeared mostly in review articles.

John Marrack was a colourful character. Behind a shyness and apparent abruptness lay kindness and intellectual integrity. He always wanted to be an athlete and was by temperament a fighter—for seven years he was welterweight champion in the London University boxing tournaments. Throughout the whole of his adult life he was a keen walker (he knew Dartmoor intimately) and he never drove where he could go by bicycle. On more than one occasion when roused to righteous anger he took the law into his own hands and used his fists: once to apprehend a thief in the laboratory and again to despatch a gang of hoodlums who misguidedly attacked him on Whitechapel Station. His war record in the RAMC—DSO as a line medical officer and MC for investigations on the poison gas used

against the British Army in 1917—illustrates this aspect of his character. So also does his consistent championship of the underdog.

During the Civil War in Spain, he was an active member of the Spanish Medical Aid Committee, and visited the International Brigade and the Spanish Republican army. About this time he became deeply concerned about the nutrition of children in Britain, influenced by L. J. Harris and Jack Drummond, and spent much time and effort campaigning for the Children's Nutrition Council—to such good effect that he was adviser to the Ministry of Food during the Second World War and wrote in 1942 a book (*Food and Planning*) which influenced the post-war planning of nutrition.

These activities were regarded by many of his contemporaries as indicating that he was finished with research, but were entirely consistent with his character. When he returned to the laboratory in the Department of Pathology at Cambridge in 1952, his main work had, in fact, been completed, but he began to exploit the growing knowledge of the structure of antibodies while devoting most of his energy to editing, for its first ten years, virtually single handed, the new journal *Immunology*. By now the importance of his earlier work had become widely understood and recognised and at the age of 76 he was made Visiting Professor at the University of Texas. At the First International Congress of Immunology in 1971 he was one of five to receive the Distinguished Service Award "For revolutionary ideas that have become commonplace in his lifetime, and for pioneering work in the physicochemical interpretation of antigen-antibody interactions".

J. H. Humphrey

Marie Laura Violet Gayler, March 1891–August 1976, was the youngest of five daughters of Mr William Gayler, Director of Stamps and Excise at Somerset House. Her mother was an artist, a Gold Medallist of the Slade School, whose paintings were often exhibited at the Royal Academy. Marie graduated from London University in 1912, and after teaching botany at the Colstan Girls' School, Bristol, in 1915 she joined Walter Rosenhain's scientific staff in the Metallurgy Department of the National Physical Laboratory, and later married Dr J. L.

Haughton, a member of the same Department. She retired in 1947.

Marie Gayler and a physical chemist, Miss I. H. Hadfield, were the first women to be appointed to the scientific staff of the Department. She became a distinguished member of Rosenhain's team, which in the 1920s and 30s at the NPL, helped to lay the scientific foundations of physical metallurgy and to give this country the leading position which it enjoyed in the subject for a couple of decades or more.

Marie Gayler's outstanding contribution, with Hanson and Haughton,

was the elucidation of the mechanism of age-hardening in the duralumin family of aluminium alloys, which had been developed empirically by Wilm in Germany. A very important outcome of the NPL work was Y-alloy, an aluminium alloy which contained nickel as well as copper, magnesium and silicon, the normal alloying elements in duralumin. The presence of nickel greatly improves the strength and hardness of age-hardened duralumin at temperatures of 150–200 °C. This makes Y-alloy eminently suitable as a material for the pistons of internal

combustion engines. Many derivatives of Y-alloy have been developed, among which are the RR series of alloys, one of which is used in the skin of the Concorde which is heated by passage through the air at supersonic speeds.

In the 1930s and 40s Marie Gayler took over the work at the NPL on dental amalgams. To understand the mechanism of the setting and hardening of amalgams she followed the traditional NPL procedure, which was to establish the metallurgical constitution of the alloys, consisting essentially of silver, tin and mercury. The setting proceeds by diffusion at room temperature of the mercury into the powdered silver-tin alloy. Novel metallographic techniques had to be worked out for the

study of the complex processes of diffusion and reaction. To ensure the satisfactory prosthetic performance of the amalgam filling, close control of the volume changes occurring during and after setting must be assured. In recognition of this side of her work Marie Gayler was made an Honorary Member of the British Dental Association in 1947.

Although her principal researches were concerned with aluminium alloys and dental amalgams, Marie Gayler also carried out important investigations on iron-manganese alloys, and on the melting points of pure silicon and iron. In 1947 the Institute of Metals, in whose Journal many of Marie Gayler's papers were published, awarded its

Platinum Medal jointly to her and her husband, Dr J. L. Haughton.

After her retirement she was able to devote more time to her interest in sculpture; she sculpted the head of the late Professor Hume-Rothery; which now stands in the library of the Department of Metallurgy in Oxford University.

Marie Gayler readily absorbed and whole-heartedly transmitted Rosenhain's enthusiasm and scientific resourcefulness. She worthily maintained and enhanced the traditions of the Metallurgy Department of the NPL, and her surviving colleagues remember her as a warm and helpful colleague of striking and attractive appearance.

A. J. Murphy

announcements

Meetings

November 11, **Ecdysone Workshop**, London (Dr. D. L. Whitehead, Tsetse Research Laboratory, Department of Veterinary Medicine, Langford House, Langford, Bristol BS18 7DU, UK).

January 29–30, 1977, **Human Skin Banking**, Milwaukee, Wisconsin (Ralph M. Guttman, MS, Director, St Mary's Skin Bank, PO Box 503, Milwaukee, Wisconsin 53201).

March 2–4, 1977, **Cell Differentiation and Neoplasia**, Houston (Stephen C. Stuyck, MD, Anderson Hospital and Tumor Institute, Houston, Texas 77030).

March 28–April 1, 1977, **Scanning Electron Microscopy**, Chicago (Deadline for abstracts: October 25) (Om Johari, Annual SEM Symposia, IIT Research Institute, 10 W 35th Street, Chicago, Illinois 60616).

May 3, 1977, **Crop Protection**, Ghent (Prof. Ir. R. H. Kips, Chairman of the Organising Committee, International Symposium on Crop Protection, Faculteit van de Landbouwwetenschappen, Coupure Links 533, B-9000 Ghent, Belgium).

May 17–21, 1977, **Study of Macromolecules by NMR**, Grasmere, UK (Dr D. H. Richards, Explosives Research and Development Est., Non-metallic Materials Branch, Powdermill Lane, Waltham Abbey, Essex).

July 11–14, 1977, **Gas Kinetics**, Manchester (Fifth International Symposium on Gas Kinetics, UMIST, PO Box 88, Manchester M60 1QD).

July 11–15, 1977, **Organometallic and Co-ordination Compounds of Ger-**

Person to Person

A limited number of copies of the *Genetic Hazards to Man from Environmental Agents*, the proceedings of a symposium held in Ottawa in May, 1975, and published in *Mutat. Res.*, **33**, (1), (1975) are available to scientists who would not have access to it otherwise. Please write to Dr John A. Heedle, Department of Biology, York University, 4700 Keele Street, Downsview, Ontario M3J 1P3, Canada.

I am conducting experiments on the variations of fungal pathogens of rice induced by pesticides. Those who are working on similar topics may please contact me if interested in exchanging views on this subject (Dr S. Balakrishnan, Dept of Pathology, College of Agriculture, Vellayani PO, Trivandrum, Kerala, India).

There will be no charge for this service. Send items (not more than 60 words) to Martin Goldman at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

manium, Tin and Lead, Nottingham (Dr P. G. Harrison, Secretary, 2nd Ge, Sn and Pb Conference, Department of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK).

August 8–19, 1977, **Physics of Quantum Electronics**, Telluride, Colorado (Professor S. F. Jacobs, Rt. 2, Box 732D, Tucson, Arizona 85715).

August 21–26, 1977, **Singlet Oxygen and Related Species in Chemistry and Biology**, Pinawa (D. Fundytus, Conference Secretary, Technical Information Services, Whiteshell Nuclear Research Establishment, Atomic Energy of Canada Ltd, Pinawa, Manitoba, Canada, ROE 1LO).

August 30–September 3, 1977, **Fourth European Crystallographic Meeting**, Oxford (Dr C. K. Prout, Chemical Crystallography Laboratory, 9 Parks Road, Oxford OX1 3PD, UK).

September 5–10, 1977, **Biology of Connective Tissue**, Uppsala (Professor T. Laurent, Biomedical Centre, University of Uppsala, Box 575, S-751 23 Uppsala, Sweden).

September 12–16, 1977, **Bioindicators Deteriorationis Regionis**, Liblice, nr Prague (Ústav krajinné ekologie CSAV honice, Czechoslovakia).

September 25–October 11, 1977, **Kimberlite**, Santa Fé, New Mexico (2nd (Ing. J. Spálény CSc.), 252 43 Prů-International Kimberlite Conference, Sylvia-K Inc., 5671 Blue Saga Drive, Littleton, Colorado 80123).

October 11–13, 1977, **Oceanic Fronts**, New Orleans, Louisiana (Deadline for abstracts: November 1) (American Geophysical Union, 1909 K Street, N.W., Washington, D.C. 20006).

November 14–18, 1977, **Pan American Conference on Forensic Applications of Medicine, Dentistry, Pathology and Palaeopathology**, Mexico City (Dr William G. Eckert, Laboratory, St Francis Hospital, Wichita, Kansas 67214).